

Directions

in Discovery

This installment of "Directions in Discovery" examines recent strategies for coupling liquid chromatography and capillary electrophoresis with matrix-assisted laser-desorption mass spectrometry.

Coupling Liquid-Phase Separations and MALDI-MS

Proteomic studies today stand at the forefront of the drug discovery process (1). The determination of which proteins are up- or down-regulated in response to disease states provides key information for selecting targets for therapeutic intervention. Characterizing the structure and function of candidate proteins permits the intelligent design of therapeutic agents.

Expression proteomics seeks to elucidate the quantitative expression patterns of proteins in a cell or tissue under defined metabolic states. The core technologies for expression proteomic studies have been two-dimensional (2-D) gel electrophoresis to separate cellular proteins, proteolytic digestion of individual proteins, and analysis of the peptide products by mass spectrometry (MS) (2). Peptide mass and sequence information can be used for database searches to establish protein identity. The success of 2-D gel electrophoresis in proteomic experiments is limited by its bias toward high-abundance proteins. To investigate the low-abundance proteins that could be key players in cell regulation, proteomics researchers are turning to 2-D column liquid chromatography (LC) separations, either in concert with or as an alternative to 2-D gel electrophoresis. Complex peptide mixtures are prefractionated by off-line or on-line ion-exchange chromatography, and fractions are separated further by capillary reversed-phase chromatography.

The preferred detection system for LC-MS of peptides has been electrospray ionization coupled with MS or MS-MS. The use of electrospray ionization permits continuous sampling of the high performance liquid chromatography (HPLC) eluent and efficient production of pseudo-molecular ions. The electrospray ionization source can be interfaced with triple quadrupole, ion-trap, quadrupole time-of-flight (TOF), TOF-TOF, or Fourier transform (FT) mass analyzers to obtain peptide mass and sequence data. A drawback of on-line

LC-electrospray ionization MS is the limited time available for data-dependent mass analysis. In a typical LC-MS measurement, analysts perform a full scan across the mass range and then select as many as five precursor ions for MS-MS analysis to obtain the sequence information. The time required for each MS and MS-MS duty cycle can compromise the amount of information captured during elution of narrow capillary HPLC peaks. This limitation can be circumvented by lengthening the chromatographic run or by stopped-flow peak-parking approaches (3). However, the extended analysis times will reduce throughput.

An alternative approach is depositing the column eluent onto a solid surface and performing off-line MS analysis. The most familiar embodiment of this approach is deposition of the sample on a stainless steel target plate, followed by matrix-assisted laser-desorption ionization (MALDI). MALDI-TOF MS has been used for the direct analysis of protein spots from 2-D gel electrophoresis. The technique is limited by the presence of interfering salts, buffers, and detergents from the gel, so desalting steps often are necessary. In addition, the presence of high-abundance components in the sample can suppress the ionization of low-abundance analytes. Coupling a front-end separation technique will minimize both of these problems. Moreover, off-line analysis relieves the time constraints of on-line LC-MS-MS. An analyst could prescreen the entire sample set from a chromatographic separation (for example, using MALDI-TOF MS) and select only the fractions of interest for additional MS-MS analysis (for example, using quadrupole TOF or TOF-TOF instruments). After they are deposited on a target plate, the peptide samples typically are stable for extended times, so repeat analysis can be performed weeks after sample acquisition.

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The advantages of MALDI-TOF MS are particularly appealing in the case of quantitative differential expression studies using isotope-coded affinity tags (4). Isotope-coded affinity tag experiments using LC-electrospray ionization MS-MS are limited by on-the-fly selection of precursor ions for MS-MS based upon ion intensity. Hence, the isotope-coded affinity tag data set tends to be cluttered with isotope-coded affinity tag-labeled peak pairs of similar intensity and no difference in expression. In contrast, MALDI quadrupole TOF MS permits selection of only those isotope-coded affinity tag peak pairs that exhibit significant differential expression for MS-MS analysis. This ability reduces database search time and increases throughput. Another advantage of MALDI-TOF for isotope-coded affinity tag studies is the predominance of singly charged molecular ions, which simplifies quantitation compared with electrospray ionization MS-MS, in which peptides can be present in multiple charge states. The high mass resolution and mass accuracy of modern TOF mass analyzers enhance database searches for protein identification.

Several approaches for coupling liquid-phase separations to MALDI-MS have been described, and this month's installment of "Directions in Discovery" will explore recent strategies for LC-MALDI-MS and capillary electrophoresis (CE)-MALDI-MS.

Fraction Collection

The simplest and most straightforward approach to interfacing a liquid separation technique with MALDI-MS is to collect fractions from the eluent stream into wells

or directly onto the target plate. The matrix solution can be added to the fraction as a separate step or can be deposited onto the target before sample introduction. In the latter case, it is convenient to include an internal standard with the matrix solution for calibration of the mass spectrometer. Hsieh and co-workers (5) collected fractions from a reversed-phase capillary HPLC separation directly onto a stainless steel MALDI target; they introduced the matrix by postcolumn addition at 60 nL/min through a capillary mounted adjacent to the exit of the separation capillary.

Miliotis and colleagues (6) describe the use of a piezoelectric flowthrough microdispenser for depositing the eluent from a capillary HPLC column onto a MALDI target. Their device generated 60-nL droplets and was operated optimally at 15 Hz. The MALDI target was precoated with a solution of matrix and nitrocellulose using a novel air-brush technique. These precoated targets were stable for several weeks either before or after sample deposition.

Collecting fractions from a CE separation can be problematic because of the extremely low flow rates delivered by separation capillaries. Johnson and co-workers (7) used a liquid sheath flow to form droplets at the capillary exit and help transport separated analytes from the capillary. Introduction of the sheath flow resulted in a 40-fold dilution of the CE eluent. This dilution was offset by the use of a prestructured sample support (an Anchor plate from Bruker Daltonics [Billerica, Massachusetts]) that comprised a hydrophobic surface with hydrophilic anchor regions arrayed on the surface, and the matrix was deposited on the anchor points before use.

Containing the collected fractions within the anchor spots during solvent evaporation concentrated the sample. A fundamental limitation of collecting fractions as discrete drops is the potential for the loss of chromatographic resolution.

An automated capillary LC system that deposits the column eluent on MALDI target plates is available from LC Packings (Sunnyvale, California). Fractions are deposited by a microfraction collector, and the MALDI matrix solution is added coaxially at the needle tip (8).

Electrospray Deposition

Lou and van Dongen (9) reported the use of electrospray deposition in the analysis of organic polymers by size-exclusion chromatography and MALDI-TOF MS. They used a microbore column with chloroform as a mobile phase delivered at 15 $\mu\text{L}/\text{min}$. The matrix solutions (prepared in chloroform-tetrahydrofuran) were introduced coaxially by a postcolumn syringe pump. The electrospray capillary was operated at 2–4 kV. Because of the high volatility of the mobile phase and matrix solutions and because of the small droplets generated by electrospray, solvent evaporation was rapid and homogeneous spots were obtained on the target.

Electrically Mediated Deposition

Ericson and colleagues (10) recently described a pulsed, electrically mediated system for noncontact deposition of the HPLC eluent onto MALDI targets. In their approach, the eluent was delivered to the tip of a grounded fused-silica capillary, which was positioned 2–5 mm from the MALDI target. They applied a 20-ms negative voltage pulse (–2 kV) to the target stage, which caused polarization of the droplet formed at the capillary tip, which in turn was pulled to the target surface by the electric field (Figure 1) (10). The pulsed nature of the electric field and the low field strength at the droplet surface prevented electrospray deposition, which would have distributed the column eluent over a wider area. Confinement of the eluent to a smaller area permitted the deposition of high-density arrays. In the authors' application, this confinement was important because the interface was configured as a four-channel system coupled with four capillary HPLC columns operated in parallel as a high-throughput platform. They used Bruker Anchor plates as the MALDI targets. When they used dihydroxybenzoic acid as matrix, it was

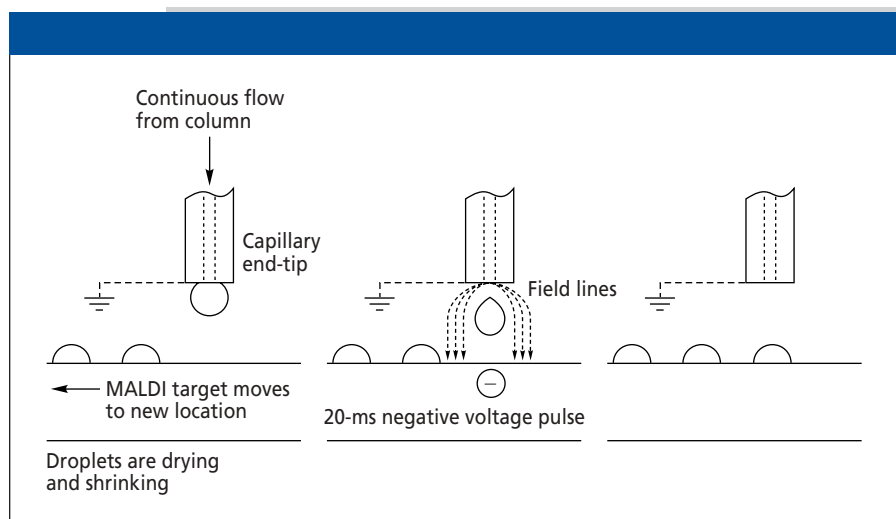


Figure 1: Principle of electrically mediated liquid deposition. (Reprinted from reference 10 with permission from the American Chemical Society.)

codeposited with the column eluents using postcolumn syringe pumps coupled by Y connections. The more hydrophobic α -cyano-4-hydroxycinnamic acid matrix was precoated onto the hydrophilic spots on the plates. By using the multichannel deposition system to precoat the matrix, they could deposit 1536 positions in less than 1 min.

Rotating Ball Inlet

Ørnsnes and co-workers (11) described an interface for continuous introduction of a flowing liquid into a MALDI-TOF MS. This interface is a modification of the rotating ball inlet developed for electron ionization. It has a stainless steel ball that is positioned in the MS vacuum chamber and rotated by a drive shaft connected to a motor (Figure 2) (11). The sample-matrix solution is deposited on the surface of the ball from a capillary. The ball rotates past a polymer gasket, and volatile components in the sample trace evaporate in the vacuum region. Because the inlet can operate continuously for several hours, it should be compatible for interfacing with capillary LC and CE separations. To date, the system is limited by poor resolution due to the relatively high source pressure (5×10^{-5} torr) and to nonparallel fields in the source. Irregularities in the ball surface also cause variations in signal intensity.

Continuous Vacuum Deposition Interfaces

A series of continuous vacuum deposition interfaces have been developed in Karger's laboratory (12–15). The first design used a rotating quartz wheel onto which the liquid was deposited from a fused-silica capillary, which was kept in contact with the

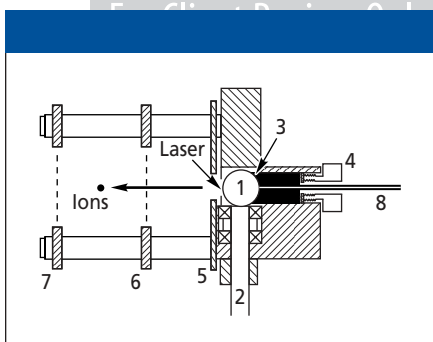


Figure 2: Diagram of the on-line rotating ball inlet-MALDI probe. 1 = 10-mm diameter stainless steel ball, 2 = drive shaft, 3 = gasket, 4 = adjustment screw, 5 = repeller, 6 = extraction grid, 7 = ground grid, 8 = capillary. (Reproduced from reference 11 with permission from the American Chemical Society.)

wheel (12). As the wheel rotated, deposited sample was transported into the MALDI ion source (Figure 3) (12). Infusion experiments with sample-matrix solutions demonstrated that a uniform track of 40- μm width and approximately 70-nm thickness was deposited on the quartz substrate. With a track width of 40 μm and a step length of 87 μm , the 100 $\mu\text{m} \times 100 \mu\text{m}$ laser spot overfilled each sample segment, which resulted in maximum sample utilization and high sensitivity. The movement of the wheel during deposition prevented the accumulation of solvent at the capillary outlet. The wheel was held at room temperature and acted as a heat reservoir to prevent solvent at the capillary outlet from freezing during evaporation in the vacuum. This interface was coupled with a CE separation capillary using a liquid junction that contained the matrix solution, which was mixed with the analyte in the infusion capillary before deposition. Drawbacks of this design included limited sampling time (the wheel was typically operated at 0.33 rpm for deposition) and the need to clean the wheel after each use.

An improved version of the continuous vacuum deposition interface by Karger's group used a disposable moving tape onto which sample was deposited from the infusion capillary (Figure 4) (13). The 80 m long \times 4 mm wide Mylar tape was transported from its cartridge, past the infusion

capillary, and through a tape guide mounted in the repeller within the ion source chamber. At a typical tape velocity of 1 mm/s, an 80-m roll lasted a full day of continuous operation. Rapid evaporation of the solvent from the tape produced a uniform sample track of 60- μm width. The moving-tape interface was coupled with a CE system using the same liquid junction described for the rotating quartz wheel interface. Because of the tape's properties, the solutions deposited onto the tape needed to contain at least 30% methanol to prevent freezing of the solution at the capillary tip. Apparently, the hydrophobicity of the Mylar surface reduced the efficiency of solution transfer from the capillary tip, and vaporization of the liquid remaining at the tip caused freezing.

The moving tape interface has been expanded for high-throughput applications by replacing the single infusion capillary with a 10-capillary array (14). Eight of the 10 infusion capillaries were used to deposit sample traces simultaneously on the advancing tape. The positions of eight sample traces were determined with a fast-scanning mirror, and samples were desorbed with a kilohertz laser. Because the sample traces were less than 80- μm wide and separated by 70 μm , a specific trace could be fully irradiated with a 100-mm laser spot without irradiating an adja-

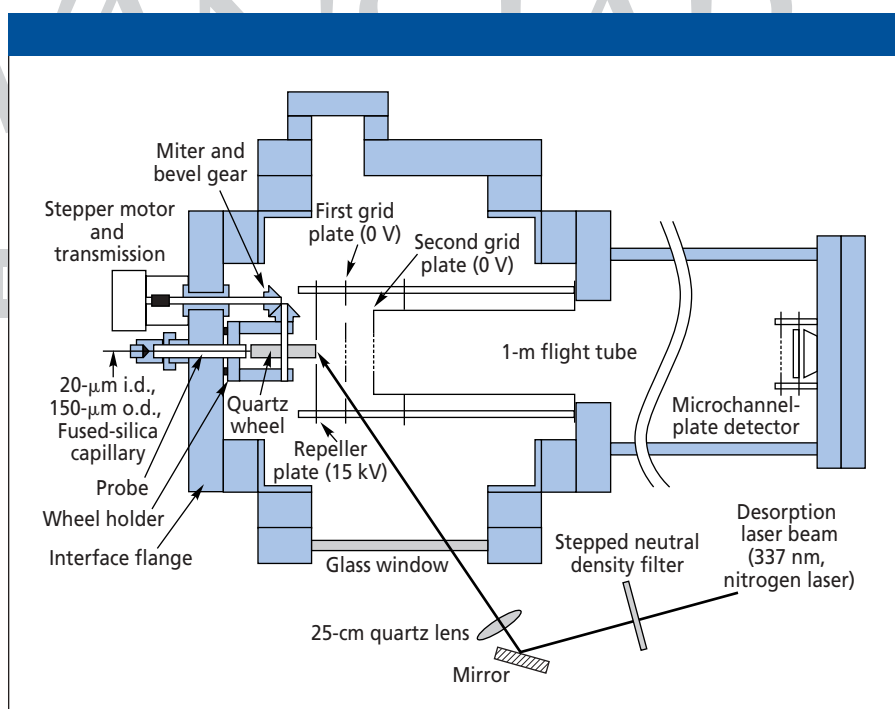


Figure 3: Schematic of a MALDI-TOF MS instrument with a continuous on-line vacuum deposition inlet incorporating an infusion capillary interfaced with a rotating quartz wheel. (Reproduced from reference 12 with permission from the American Chemical Society.)

cent trace. The high repetition rate of the laser permitted the target with the array of deposited traces to be moved in one dimension and scanned with the desorption laser by the fast-scanning mirror in the perpendicular dimension. The infusion capillary array was interfaced with eight separation capillaries using a common liquid junction.

The vacuum deposition approach has been implemented by Karger's laboratory in a stand-alone rough vacuum chamber in which separation streaks are deposited on conventional stainless steel MALDI targets (15). As in the on-line vacuum deposition designs, the width of the deposited sample trace (100 μm) was sufficiently smaller than the laser spot size (150 μm) to allow the beam to encompass the entire trace for good sample utilization. This setup, coupled with fast solvent evaporation and even distribution of sample across the trace, produced improved signal reproducibility. To accommodate a 1-h sampling time, they deposited the sample on the target in a serpentine streak. With the infusion capillary fixed at a 60° angle, the target was driven in one direction, and the deposition probe was rotated 180° near the end of the plate with the target moving perpendicular to the direction of the previous deposition to offset the new trace. This off-line vacuum deposition system was interfaced with a

CE separation capillary in a manner similar to that described for the on-line deposition systems; that is, with a liquid junction.

Rejtar and colleagues (16) recently modified this deposition system to enable deposition of both continual streaks and discrete droplets. The deposition procedure was improved by spin-coating the target plate with nitrocellulose before sample deposition. This modification permitted the deposition to be conducted at a higher pressure (200 torr versus 1 torr), which resulted in slower drying and the formation of microcrystals similar to those observed in conventional dried-droplet methods. This system was configured for use with four nano-LC columns operated in parallel. In an additional modification to the system, Chen and co-workers (17) deposited a five-component peptide mix adjacent to the analyte tracks for use as an external calibrant.

Continuous Off-Line Atmospheric-Pressure Deposition

Several researchers have accomplished off-line atmospheric-pressure deposition of liquid-phase separations. Zhang and Caprioli (18,19) coupled CE with atmospheric-pressure deposition. In their system, the CE capillary was held in direct contact with the target plate, which carried a cellu-

lose membrane precoated with matrix. The stainless steel body of the target plate was connected to the ground of the CE high voltage power supply. The background electrolyte was eluted from the separation capillary by electroosmotic or hydrodynamic flow as the moveable target advanced and formed a narrow sample track.

Wall and colleagues (20) described a continuous atmospheric-pressure deposition system for HPLC. In their system, eluent from the reversed-phase HPLC column was introduced into a heated nebulizer to produce a fine spray of droplets that rapidly evaporated on the MALDI target. This target was a disposable plate precoated with matrix before use. The desolvation system was compatible with eluent flow rates ranging from 200 nL/min to 50 $\mu\text{L}/\text{min}$, which allowed use with nano-, capillary, and microbore LC separations. The deposition pattern was generated by a motorized x - y translation system that controlled the target position. Continuous deposition preserved chromatographic integrity, with sensitivity and resolution determined by drive speed. Deposition of a 200-min gradient at 10 $\mu\text{L}/\text{min}$ was demonstrated using a series of eight 45-mm tracks in a discontinuous serpentine pattern. This system has been commercialized by Waters Corp. (Milford, Massachusetts) as a sample collection module that includes a heated capillary nebulizer and an x - y stage holding the precoated MALDI target.

Summary

This "Directions in Discovery" column described various methods for coupling CE and HPLC separations with MALDI. Fraction collection, either off-target or directly onto the MALDI target, is the most straightforward method and is readily implemented with commercially available robotic collection systems. However, chromatographic resolution can be compromised. Alternatively, the eluent can be sprayed or deposited onto the target either as a continuous stream or discrete droplets. The MALDI matrix can be mixed with the column eluent by postcolumn addition, precoated onto the target before use, or added after sample deposition. Both atmospheric-pressure and vacuum deposition systems have been developed. Most LC- and CE-MALDI interfaces operate off-line. Decoupling the separation and MALDI-MS relieves the time constraints on MS data acquisition and reanalysis.

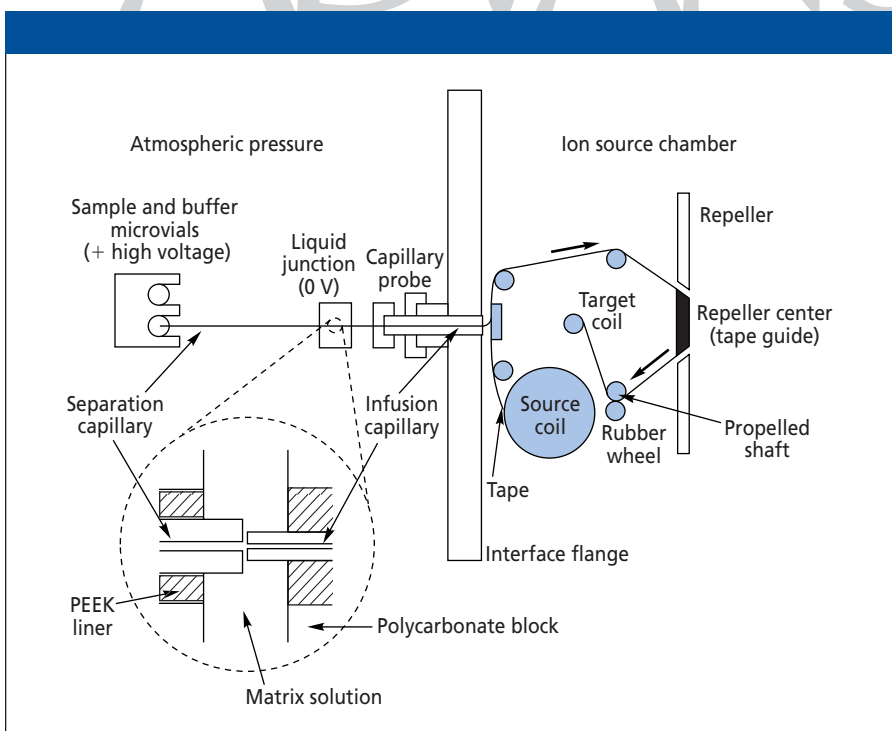


Figure 4: Schematic of a continuous on-line vacuum deposition inlet incorporating an infusion capillary, liquid junction, and moving tape interface. (Reproduced from reference 13 with permission from the American Chemical Society.)

On-line coupling of the separation with MALDI-MS permits direct acquisition of sample information for high-throughput environments; both single-channel and multichannel approaches have been reported. To date, no on-line coupling technique has been commercialized.

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