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Technical Notes

Electrophoresis Separation in Open Microchannels. A Method for Coupling Electrophoresis with MALDI-MS

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The separation of biological mixtures in open microchannels using electrophoresis with rapid and simple coupling to mass spectrometry is introduced. Rapid open-access channel electrophoresis employs microchannels that are manufactured on microchips. Separation is performed in the open channels, and the chips are transferred to a matrix-assisted laser desorption/ionization (MALDI) source after the solvent is evaporated. The matrix (2,5-dihydroxybenzoic acid) is placed in the solution with the run buffer before the separation of the analyte components. After separation, the solvent is evaporated and the microchip is ready for MALDI-MS analysis. The microchip is placed directly into a specially designed ion source of an external source Fourier transform mass spectrometry instrument. Separation of simple mixtures containing oligosaccharides and peptides is shown.

The union of electrophoresis and mass spectrometry (MS) combines a separation method of general utility with a sensitive detection method that also provides molecular weight and structural information. The need for the combined methods has long been recognized, and the coupling has been attempted in various ways. The simplest approach is to use capillary electrophoresis and combine it with electrospray ionization (ESI).[1−6] However, coupling electrophoresis with MS remains fraught with difficulties so that CE/MS analysis is far from routine. The intolerance of ESI to moderate concentrations of ionic species from salts such as sulfates, phosphates and borates—necessary for electrophoresis—makes the two methods somewhat incompatible.

Matrix-assisted laser desorption/ionization (MALDI)[7] is a method that normally complements ESI. It is more tolerant of samples that are not necessarily "clean". It is often used with samples that contain various ionic species including salts. However, the direct mating of electrophoresis and MALDI has its own technical limitations. While one is a dynamic fluid system, the other is static and crystalline. With exceptions such as the use of liquid matrixes,[8] MALDI usually requires the cocrystallization of the sample with a matrix that acts as a chromophore for MALDI. For this reason, attempts to mate CE with MALDI directly have met only limited success;[9] albeit the use of a vacuum deposition device[10] and piezoelectric microdispensers[11] for producing MALDI sample from CE eluant shows considerable promise. The analysis of electrophoresis eluant off-line provides the most practical method for coupling with MALDI.[12−16]

In this report, we present the concept of rapid open-access channel electrophoresis (ROACHE) where mixtures are separated in open microchannels cut into glass microchips. The use of microchips for analysis is one of the most exciting advancements in separation technology.[17−23] They also provide a direct method

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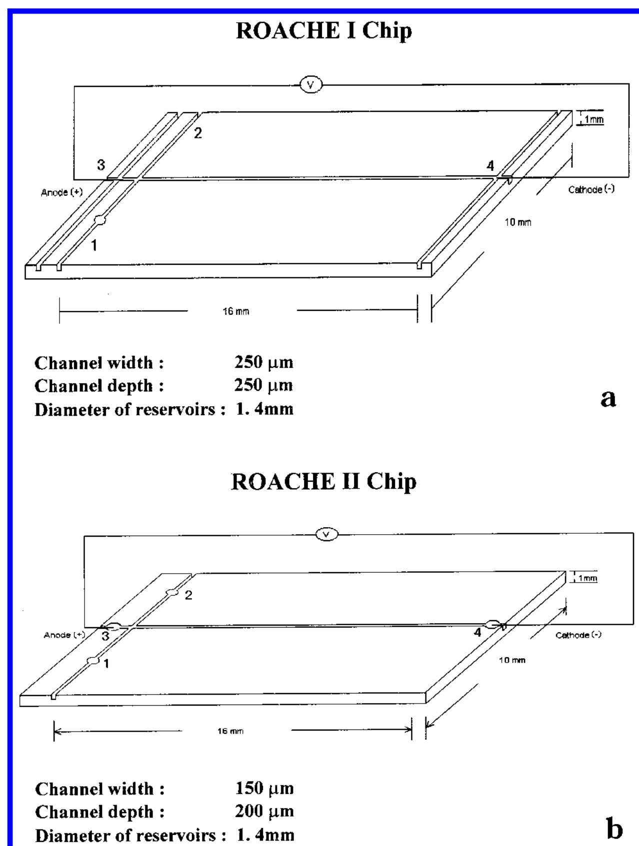

Figure 1. Schematics of ROACHE I (a) and ROACHE II (b) chips. The channels are open for exposure to the MALDI laser beam.

for coupling capillary electrophoresis with mass spectrometry through electrospray ionization. [5,24,25] Microchips have not been used to couple electrophoresis with MALDI-MS. In ROACHE, matrix is added to the buffer solution before the separation. When the solvent is evaporated at the end of the separation, the channel yields a MALDI sample complete with matrix and ready for analysis. The laser is then used to raster the channel to provide mass spectra as a function of channel position. The electroosmotic movement of peptides and oligosaccharides is illustrated as well as the separation of a mixture containing a peptide and an oligosaccharide.

EXPERIMENTAL SECTION

Chemicals. The oligosaccharides and [Lys[1]]-bradykinin were obtained from Sigma (St. Louis, MO). The matrix 2,5-dihydroxybenzoic acid (DHB) was obtained from Aldrich (Milwaukee, WI). Acetic acid and ammonium hydroxide were obtained from Fisher Scientific (Fairlawn, NJ). All compounds were analytical grade and used without further purifications. Permethylated maltohexaose was synthesized in our laboratory from the parent (maltohexaose, Sigma) using the procedure by Ciucanu.[26]

Microchip Fabrication. Microchips were fabricated in the U.C. Davis microfabrication facility. The schematic layouts of the ROACHE microchips are shown in Figure 1. These chips were made from glass that were 2 cm long, 1 cm wide, and 1 mm thick. Channels were cut into the chip using a wafer saw. The reservoirs were formed using a diamond tip drill bit.

Sample Injection Devices. Two types of injection methods were employed: electroosmotic (electrokinetic injection) and hydrodynamic. Electroosmotic injection involved applying voltages on the cross channel to move the substance electrophoretically. After an amount of sample intercepted the main channel, the voltages were reconfigured to draw most of the sample back into the sample reservoir and to the sample waste, leaving a small amount of sample at the top of the separation channel.[19]

A device was constructed to perform hydrodynamic injection. Because the channels were open, hydrodynamic injection was performed by creating a bead on the end of a syringe. This syringe was placed on an *x,y,z*-translational stage. The droplet was directly placed on the beginning of the main separation channel. To minimize the drop size and keep the sample amounts more consistently, a fused-silica tube (25-mm i.d.) was fitted to the end of the syringe. Two cameras were employed to monitor the droplet size and the injection position on the main column. This mode of sample introduction produced significantly better reproducibility during the separation.

Electrophoresis Separation. Before each separation, a 1 M NaOH solution was applied to the channel for 1 h. This was followed by several rinses with deionized water. A buffer solution (see below) was then applied to the channels before the sample injection. The chips were reused by first rinsing them with 1% HCl solution followed by washings with ethanol and deionized water. To perform the separation, Pt wire was affixed either to the reservoir (ROACHE chip II) or the end of the column (ROACHE chip I) for electrical contact. During the separation, the chip was placed on an acrylic housing that allows it contact with an aluminum block. The aluminum block was milled out to allow temperature-controlled water to flow through the block. After the separation period, the solvent was evaporated using forced air at ambient temperature from a heat gun or by vacuum.

MALDI-FTMS Analyses. Experiments were performed using a custom-built external source FTMS described in detail in earlier publications.[27] A new ionization source was built specifically for the ROACHE chips and is described also in an earlier publication.[28]

The introduction and removal of ROACHE chips were achieved by a load-lock system that is mounted on the top of the ion source chamber. The ROACHE chip was placed on an *x,y,z*-movable stage that was installed inside the source chamber and controlled by three independent manipulators. A CCD camera equipped with a zoom lens monitored the position of the chip relative to the laser beam.

RESULTS

Effect of Sample Injection and Diffusion on Peak Width. To observe how the peak width is affected by the sample injection and diffusion during the preparation for MALDI-FTMS, sample

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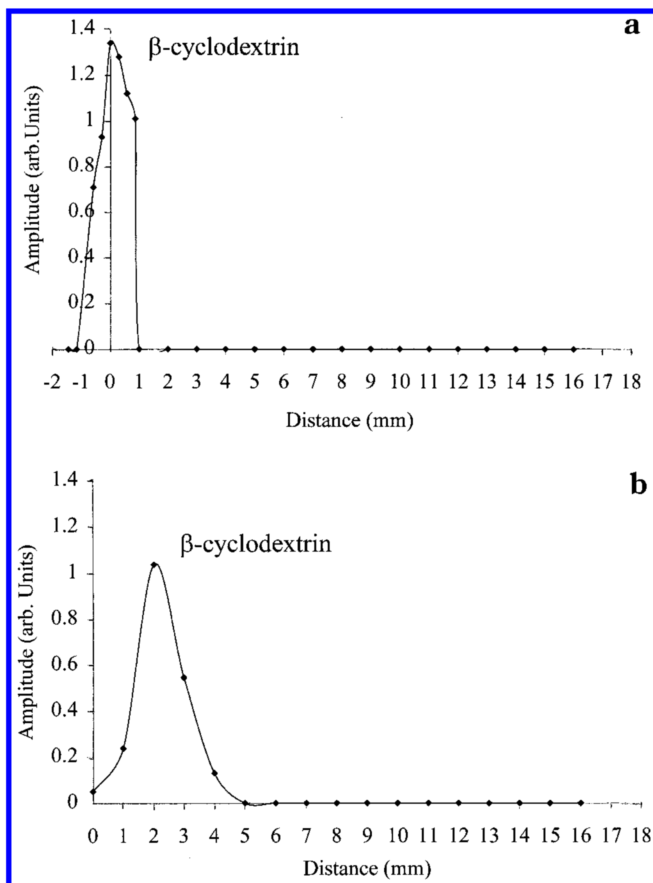

Figure 2. (a) MALDI-FTMS amplitude of the quasimolecular mass of β-cyclodextrin (m/z 1451.7) plotted as a function of column length. The position 0.0 mm corresponds to the point of sample injection. No voltage was applied to the column. (b) Same experiment with voltage of 400 V applied for a period of 10 s to the channel.

was placed on the column and prepared for MALDI-FTMS analysis in the absence of an electric field. The channels (250 μm × 250 μm, ROACHE I chip) were first filled with approximately 4−5 μL of the run buffer. The run buffer was produced by dissolving 9 mg of DHB in 30 μL of ethanol. This amount was added to 35 μL of CH_3CO_2H (0.18 M in H_2O) and 35 μL NH_4OH (0.26 M in H_2O). The resulting DHB concentration was ~90 mg/mL. Introducing buffer to channels with column diameters of 250 μm or greater involved simply placing, via syringe, buffer at any point in the column and allowing capillary action to fill the entire channel. Immediately thereafter, ~0.1 μL of the analyte, permethylated β-cyclodextrin, 1.0 mg/mL in 50:50 water/methanol, was placed at the injection point (0.0 mm). This process was performed hydrodynamically using a 10-μL syringe with a stainless steel needle. The volume of the analyte was estimated visually using the CCD camera. After the separation time, the solvent was removed. The microchip was then placed in the ionization source, and the laser beam was used to trace the column in increments of ~0.3 mm. The plot of the intensity of the quasimolecular ion ($[M + Na]^+$, m/z 1451.7) versus the distance from the point of injection is shown in Figure 2a. Note that the maximum of the peak is at the point of injection. There was some diffusion of the analyte producing band broadening (1.0 mm at half-height).

Observation of Electrophoretic Motion on ROACHE Microchips. To observe electrophoresis, pure analytes were again applied to the column. In a typical experiment, voltage (400 V) was applied for a period of 10 s under ambient conditions (22 °C). With ROACHE I chips, we found that the solvent overflowed near the cathode at the end of the column. However, the level of the solvent in the main column, where the separation occurred, remained largely unaffected.

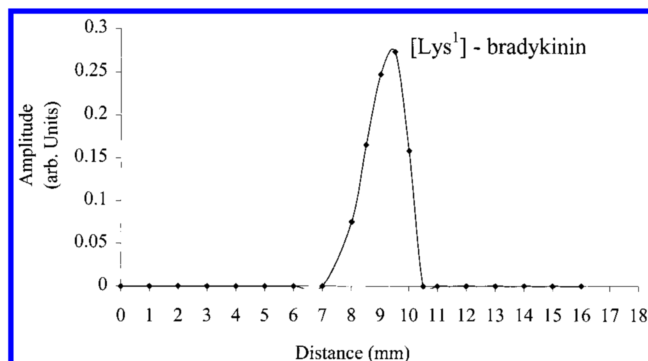

Figure 3. MALDI-FTMS amplitude of the quasimolecular ion of [Lys[1]]-bradykinin (m/z 1032.5) plotted as a function of column length. A 400-V dc voltage was applied to the channel for a period of 20 s.

Crystallization of the sample for MALDI analysis required 25−30 s. The column was again traced with the laser, and the resulting plot of the quasimolecular ion as a function of distance is shown in Figure 2b. Permethylated-β-cyclodextrin traveled in the open channel to a distance of ~2 mm. The movement was attributed primarily to electroosmotic flow generated in the channel since the sample is neutral. As observed, the peak width was slightly broader than that found in Figure 2a. The increased broadening was likely due to the additional separation time and possibly Joule heating during the electrophoresis event.

For comparison, a peptide [Lys[1]]-bradykinin (4.7 mg/mL, 1:1 methanol/water) was injected and tested on the same chip using identical conditions. A dc voltage of 400 V was again applied over a period of 20 s with an additional time of 30 s to evaporate the solvent for MALDI. The quasimolecular ion ($[M + H]^+$, m/z 1032.5) was monitored as a function of distance, and the resulting plot is shown in Figure 3. As expected, the peptide, with two positive charges under the pH 3.0 conditions, moved significantly further (9.5 mm).

Separation of Simple Mixtures with ROACHE-MALDI-FTMS. Two test samples were prepared. The first mixture (mixture A) was prepared by mixing equal amounts (0.5 mg/mL) of permethylated β-cyclodextrin and [Lys[1]]-bradykinin in ethanol/water (50:50) solvent. A second mixture (mixture B) was prepared by dissolving equal amounts (0.5 mg/mL) of [Lys[1]]-bradykinin and maltohexaose (5 mM) in ethanol/water (50:50) solvent.

Mixture A was separated using a ROACHE I chip with 250-mm channel. Electrokinetic injection was used to introduce analyte into the central channel. A dc voltage of 280 V was applied at point 1 on the chip while point 2 was held at ground for 5 s. This caused the sample to move from the reservoir (point 1) to point 2, bisecting the main column. A dc voltage of 400 V was then applied to point 3 with point 4 held to ground for ~15 s. Simultaneously, both point 1 and point 2 were fixed at 280 V to prevent the sample in the injection channel from being reintroduced into the separation channel. Forced ambient air was employed to dry the channel quickly while the voltage was in place.

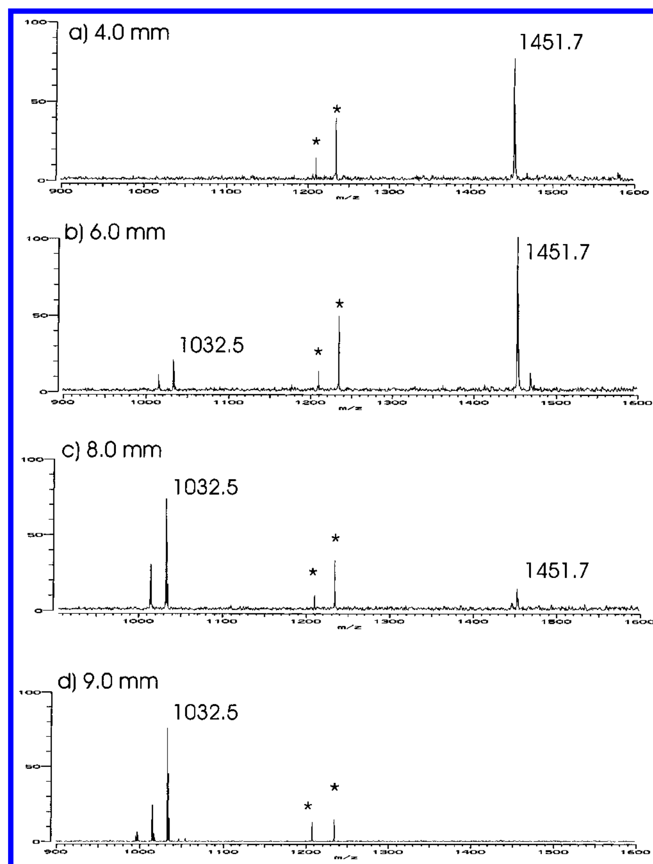

Figure 4. MALDI-FTMS spectra of a mixture containing β-cyclodextrin(m/z 1451.7) and [Lys[1]]-bradykinin (m/z 1032.5) at four different positions corresponding to (a) 4, (b) 6, (c) 8, and (d) 9 mm. A dc voltage corresponding to 400 V was applied for a period of 15 s. The asterisked peaks are due to electrical noise.

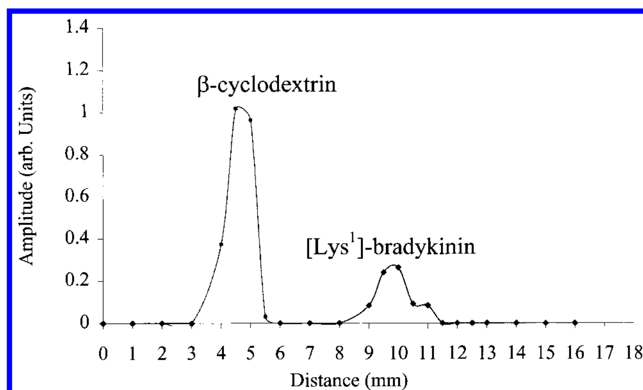

Figure 5. Electropherogram obtained on a ROACHE II chip of a mixture containing β-cyclodextrin and [Lys[1]]-bradykinin.

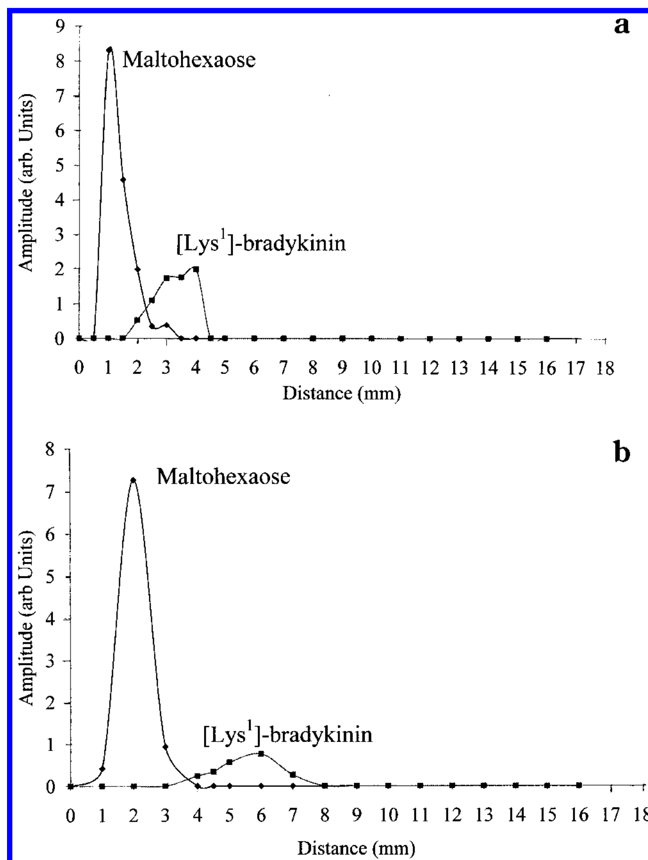

Figure 6. Electropherogram of a mixture containing maltohexaose and [Lys[1]]-bradykinin with 400 V applied to the channel and (a) 15 and (b) 20 s of separation times.

Figure 4 shows a series of mass spectra corresponding to four positions (4, 6, 8, and 9 mm) on the chip in the experiment described above. The distances are measured from the crossing of the channels (0 mm). At 4 mm, a strong signal was obtained corresponding to m/z 1451.7, the sodium complex of permethylated β-cyclodextrin (Figure 4a). No signal was detected corresponding to the [Lys[1]]-bradykinin at this point. The signal intensity of the permethylated β-cyclodextrin increased to the maximum at 6 mm (Figure 4b). At this point, a weak signal for [Lys[1]]-bradykinin ($[MH]^+$) also appeared at m/z 1032.5. At 8 mm, the intensity for [Lys[1]]-bradykinin increased while the signal corresponding to permethylated β-cyclodextrin decreased further (Figure 4c). Finally, the [Lys[1]]-bradykinin signal was most intense at 9 mm while permethylated β-cyclodextrin was now absent (Figure 4d).

Although the ROACHE I chips were relatively simple to fabricate, there were some technical problems particularly with the placement of the electrode. To maintain electrical contact, wires were placed directly on the channel but this affected the flow. For wide channels (500 μm), the solvent flow was not a problem. However for narrower channels (250 μm and below), consistency of the flow was a problem. Furthermore, heating occurred near the wires causing the solvent in the area to evaporate, thereby producing crystallization that blocked the flow. In voltages above 400 V, bubble formation around the platinum wires in the channel was also observed. For these reasons, ROACHE II chips were produced with reservoirs at the ends of both channels.

Separation was performed on ROACHE II chips. Figure 5 illustrates our best example employing a channel with width and depth of 250 μm. For this experiment, sample was introduced with the electrokinetic injection. A potential of 400 V and a 20-s separation period were used. Complete separation was achieved under ambient temperatures with [Lys[1]]-bradykinin traveling to ~10 mm and permethylated β-cyclodextrin to ~5.5 mm.

A sample containing permethylated maltohexaose and [Lys[1]]-bradykinin (mixture B) was examined on a ROACHE II chip with 150-μm channels. With a dc voltage of 400 V applied for 15 s and the temperature set to 10 °C, the mixtures were partially separated

(Figure 6a). Maltohexaose had traveled to only ~1 mm while the [Lys^1]-bradykinin traveled to almost 4 mm. With a longer separation time of 20 s, nearly complete separation was obtained (Figure 6b).

CONCLUSIONS

These experiments illustrate the concept of open channels for separation and their coupling with MALDI-MS. ROACHE is akin to gel electrophoresis without the complicating gel. In two-dimensional gel electrophoresis, compounds are separated on the basis of charge on one axis and mass on another. For the identification of proteins, a more exact mass analysis is necessary; samples are often removed from the gel and further analyzed by mass spectrometry. The ROACHE-MS couple eliminates that step and may provide an alternative to 2D gels. CE/ESI-MS provides similar information but is more transient; the relatively low duty cycles of many mass analyzers make tandem MS difficult.

Although the experiments presented in this report employed primarily 400 V, higher and lower voltages were also examined. With higher voltages (up to 800 V was studied), microbubbles appeared near the electrodes. The bubbles were probably formed by evaporation of the solvent due to Joule heating. The bubbles were often entrained in the solvent and moved with the electroosmotic flow. This action severely affected the stability of the flow as it interfered with the electrical contact between the anode and the cathode. However, we found that bubble formation was suppressed by cooling the chip (5 °C) thereby allowing higher voltages to be achieved. Similarly, decreasing the dimension of the channel also minimized bubble formation.

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