Analysis of Single Mammalian Cell Lysates by Mass Spectrometry

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Single-cell analysis can provide an insight to the detailed chemistry of a cell on individual bases, which allows exploration of the heterogeneity of the cells and the relationship to their overall biological functionality.[1−4] However, because of the small "sample" volume, trace quantity of analyte, and complex cellular matrix, the chemical analysis of single cells remains formidable. Recent advances in microcolumn separation methods, such as capillary electrophoresis (CE) and open-tubular liquid chromatography, with the arrival of miniaturized and highly sensitive detection schemes have led the way to detecting cellular components in single cells.[5−7] Among them, electrochemical and fluorescence detection systems provide the most sensitive approaches, although information on chemical identity deduced from these techniques is often very limited.

On the other hand, mass spectrometry (MS) can potentially provide chemical analysis at the cellular level with the benefit of unsurpassed chemical specificity. Indeed, a few reports on the analysis of cellular components in single large biological cells exist.[8] The analysis of cellular proteins from small populations (i.e., 5−20 cells) of human erythrocytes using electrospray ionization MS combined with CE is also reported.[9] However, the analysis of single small-volume cells has not been as successful. The major obstacles to analyzing these small cells lie in the limited sensitivity that a mass spectrometric method can provide and the requirement of special sample handling techniques for cell presentation to the mass spectrometer. We present a mass spectrometric approach for highly sensitive detection of small-volume samples. The analysis of cellular proteins from a single erythrocyte with a total cellular volume of 87 fL is shown. The success in analyzing such a tiny cell suggests that this method is applicable to the analysis of other important mammalian cells as well. We note that, in a recent conference where we presented this work, Hofstadler et al. described recent progress in CE MS including the detection of hemoglobin from single erythrocytes.[10]

Our method involves the use of matrix-assisted laser desorption ionization (MALDI)[11] for the generation of ions from the cellular components. In conventional operation, a suitable matrix is chosen to mix with the analyte in a ratio of >500:1. About 1 μL of the solution mixture is typically placed on a sample probe of about 2 mm diameter using a micropipet. The success of single-cell analysis is largely attributed to the development of a novel microspot sample deposition method as opposed to the conventional 1 μL sample delivery method. The idea of microspot MALDI is to reduce the sample presentation surface with respect to the laser desorption site and ion acceptance volume in the mass spectrometer to improve the sampling efficiency. Previous reports describing the use of chemical etching or laser drilling of a picoliter volume hole directly on the sample probe[12,13] as well as depositing a small volume sample onto cellulose membranes precoated with matrix[14] show improved sensitivity.

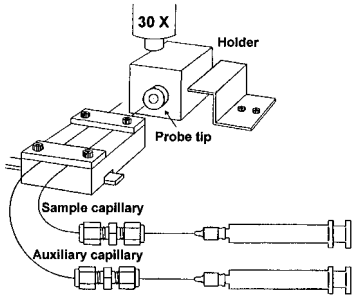

Figure 1. Microspot MALDI sample delivery system showing a sample and MALDI probe tip holder and a movable *xy* stage.

In our approach, as shown in Figure 1, a fused silica capillary for sample delivery and an auxiliary capillary are attached to a movable *xy* stage and connected to a disposable syringe. These capillaries are deactivated using a silylation fluid to reduce sample adsorption. During sample loading, the sample vial is placed on a sample holder and the *xy* stage is moved to allow the capillary to contact the sample solution. A measured volume of a sample, which is determined by the length of the sample plug in the capillary, is then withdrawn using the syringe under microscopic observation. Some air is then drawn into the capillary to minimize solvent evaporation. The sample vial is replaced with the MALDI probe that is precoated with a thin layer of a matrix. The capillary loaded with the sample is then moved to the center of the probe surface, and the sample is deposited onto the matrix layer. The sample spot is always placed in or near the center of the probe, which can be readily observed under the microscope during the delivery. Using capillaries with internal diameters from 10 to 200 μm, the volume delivered can be varied over a wide range (up to 100 nL). As little as 20 pL of sample solution can be accurately delivered onto the matrix layer producing an ~100 μm diameter spot on the probe surface. The laser desorption beam is a 50 × 180 μm oval that is prealigned with the center of the probe surface. By rotating the sample probe, MALDI mass spectra are recorded from different areas in the sample spot. A video camera is used to assist in aligning the laser with the probe center. A high-resolution time-of-flight mass spectrometer with a 1 m flight tube operated at 20 kV is used for MALDI ion detection.[15] A 1 μs time lag was used between ion desorption and extraction, and the amplitude of the extraction pulse was varied to focus the ion of interest.

The high sensitivity provided by this microspot MALDI approach can be demonstrated with the example shown in Figure 2 for substance P (oxidized form) using α-cyano-4-hydroxycinnamic acid (HCCA) as the matrix. The 4 mm diameter MALDI probe was loaded with 0.9 μL of 5 mg/mL HCCA in

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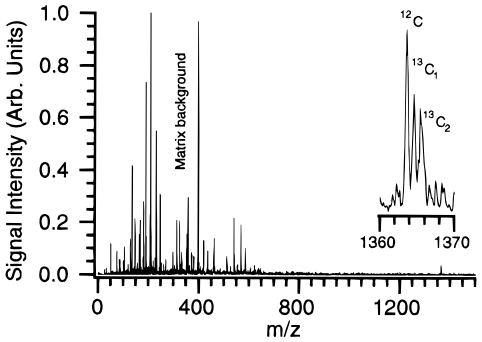

Figure 2. Mass spectrum of substance P (oxidized form, observed mass of the all ^{12}C peak $(M + H)^+ = 1363.3$ Da) from a sum of 33 single laser shots with a total sample loading of 0.97 amol.

60% methanol/acetone (v/v) to form a very thin matrix layer, followed by 0.35 μL of HCCA saturated in 25% methanol/water (v/v). The total amount of sample loaded was 0.97 amol (195 pL of a 5 nM solution). After the sample dries, very small ($\sim$1 μm) crystals formed. The total thickness of the sample/matrix layer is generally less than 5 μm. The signal-to-background-noise ratio is 16, and the mass resolution is sufficient to observe the isotope peaks. Overall, the current system provides attomole sensitivity for peptides and tens of attomoles for proteins, such as myoglobin. Further improvement of the sensitivity should be possible by optimizing the sample and matrix preparation process as well as the instrumental configuration.[16]

To illustrate the application of this method to single cell analysis, red blood cells were chosen as a model system because they are readily available and small (8 μm diameter and 2 μm thick).[1] The total cell volume is $\sim$87 fL, which is perhaps the smallest biological "sample" analyzed by mass spectrometry. In this analysis, red blood cells were preserved at 4 °C as a packed button of cells in an anticoagulant preserving solution of citrate−phosphate−dextrose. The working suspension of cells was a dilution, 0.13% by volume, of the packed button in an isotonic saline solution, 0.85% by weight in NaCl. This suspension has an erythrocyte concentration of 10−14 cells/nL. The cell suspension was loaded to a deactivated glass tube with a diameter of 1.3 mm. For handling cell suspension, the sample capillary is a 20 μm i.d. fused silica capillary with the external polyimide coating removed at one end. The external diameter was reduced to 48 μm by etching with hydrofluoric acid. Before loading the cell suspension, the capillary was filled with $\sim$0.5−1 nL of water. A variable amount of cell suspension, with the volume dependent on the desired number of cells to be analyzed, was then loaded to the capillary. For single-cell loading, the volume withdrawn from the cell suspension was $\sim$100 pL. An air gap was inserted between the water solution and the cell solution. The exact number of cells was verified using a higher microscope magnification. The cell suspension was then delivered to the probe tip that was previously covered with a thin layer of matrix (0.9 μL of 6 mg/mL sinapinic acid in 60% methanol/acetone (v/v)). During the transfer of the cell to the probe, the water in the capillary is mixed with the cell suspension, resulting in cell lysis. A second layer of matrix (20 nL of 5 mg/mL sinapinic acid in 20% acetonitrile/water (v/v)) was added on top of the sample using the auxiliary capillary. The probe tip was then loaded to the MALDI instrument for mass analysis. As a control experiment, the same sample loading procedure was followed where an aliquot of suspension with no cells present was loaded. The spectrum (not shown) displayed peaks only from sinapinic acid.

Figure 3 shows the mass spectra from loading three cells (A) and one cell (B). The spectra display two intense peaks from

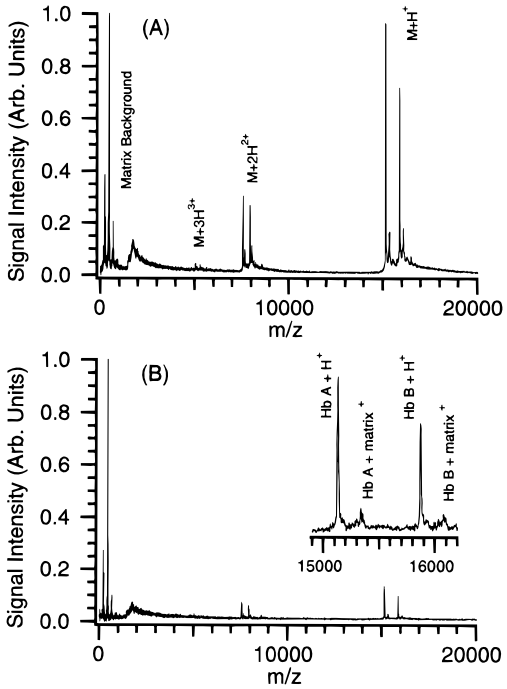

Figure 3. MALDI mass spectra of the content of human erythrocytes from (A) three cells loaded to the probe and (B) one cell loaded from a sum of 158 and 110 single laser shots, respectively.

the major components in the cell (i.e., the subunits of hemoglobin without the heme prosthetic group). Apohemoglobins A and B have masses of 15 126.4 Da (15 126.2 Da observed, error 13 ppm) and 15 867.3 Da (15 867.9 Da observed, error 38 ppm), respectively. The instrument was externally calibrated with equine cytochrome *c* (average MW of 12 360.2) and equine apomyoglobin (average MW of 16 951.5). A very similar spectrum was obtained using high-concentration hemoglobin solutions. The peaks observed below 1 kDa are from the matrix; whereas, the peaks at $\sim$7−8 and 5−6 kDa are the doubly-charged and triply-charged molecular ions, respectively. The unresolved peak at *m/z* of $\sim$2000 is from the matrix ions. A mass filter was used to deflect the low mass ions below 1.5 kDa. Considering 450 amol of hemoglobin are present in one cell,[1] it is not surprising to observe a strong signal. However, what is remarkable is that, despite the high salt content, the mass spectrum shown in Figure 3B for the single-cell analysis displays a mass resolution of 1500 fwhm (full width at half maximum). No sample cleaning was employed. The tolerance of MALDI to salts and buffers is clearly an advantage.[11]

This work illustrates that loading and analyzing a small-volume single cell is feasible by the microspot MALDI technique. The spectra and results shown are very reproducible from repeated preparations. This technique, even in its current form, should have considerable analytical utility. The addition of several other features to this technique including quantitation, affinity separation, nanoliter or picoliter chemical and enzymatic reactions,[17,18] and tandem MS should further expand the usefulness of this mass spectrometric approach. Also note that, in addition to single-cell analysis, the microspot MALDI technique should find general use in handling a small amount of material for biological applications, including the analysis of proteins isolated by polyacrylamide gel electrophoresis.

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