Identification of Mammalian Cell Lines Using MALDI-TOF and LC-ESI-MS/MS Mass Spectrometry

Xu Zhang, Mark Scalf, Travis W. Berggren, Michael S. Westphall, and Lloyd M. Smith

Department of Chemistry, University of Wisconsin-Madison, Madison, Wisconsin, USA

Direct mass spectrometric analysis of complex biological samples is becoming an increasingly useful technique in the field of proteomics. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a rapid and sensitive analytical tool well suited for obtaining molecular weights of peptides and proteins from complex samples. Here, a fast and simple approach to cellular protein profiling is described in which mammalian cells are lysed directly in the MALDI matrix 2,5-dihydroxybenzoic acid (DHB) and mass analyzed using MALDI-time of flight (TOF). Using the unique MALDI mass spectral “fingerprint” generated in these analyses, it is possible to differentiate among several different mammalian cell lines. A number of techniques, including MALDI-post source decay (PSD), MALDI tandem time-of-flight (TOF-TOF), MALDI-Fourier transform ion cyclotron resonance (FTICR), and nanoflow liquid chromatography followed by electrospray ionization and tandem mass spectrometry (LC-ESI-MS/MS) were employed to attempt to identify the proteins represented in the MALDI spectra. Performing a tryptic digestion of the supernatant of the cells lysed in DHB with subsequent LC-ESI-MS/MS analysis was by far the most successful method to identify proteins. (*J Am Soc Mass Spectrom* 2006, 17, 490–499) © 2006 American Society for Mass Spectrometry

There is great interest in characterizing the proteins present in cells. Among the many vital functions they perform, proteins catalyze a variety of chemical reactions, support a range of skeletal structures, control membrane permeability, modulate the concentration of metabolites, and control gene expression. Mass spectrometric analysis of both bacterial and mammalian cells has been a rapidly developing and important field over the last decade.

MALDI mass spectrometry [1, 2] has been applied to both the analysis of whole bacterial cells [3–6] and the analysis of proteins extracted from bacterial cells [4, 7]. Both mild aqueous acids [3, 7] and acidic MALDI matrices [3, 4, 7, 8] have been employed to extract proteins from bacterial cells. MALDI-TOF analysis of proteins extracted directly from bacterial cells without prior sample clean-up has been reported [3, 4, 7]. However, to our knowledge, this is the first report of mammalian cells analyzed directly after extraction into a MALDI matrix.

Studies involving mass spectral analysis of mammalian cells have been more extensive than those involving bacterial cells. Analyses of mammalian cells using mass spectrometry can be split into two categories—studies

involving a large number of cells and studies involving single cells. Working with a large number of cells, mass spectrometry has been employed to quantify changes in protein expression between two populations of cells using stable isotope labels [9–19], to generate diagnostic patterns denoting a disease state [20–24], and to identify proteins important in various cellular functions and pathways (i.e., differentiation, transcription, translation, ubiquitination, membrane proteins) [25–40].

Various techniques have been employed to extract information about the protein composition of cells before MS analysis. Cell lysis is a major step in sample preparation, and the choice of the lysis protocol is an important factor in the success of the subsequent assay. For mammalian cell lysis, PBS buffer [41] or lysis buffer [42, 43] followed by mechanical disruption, such as vortexing or ultrasonical disruption, are the most common techniques employed. Centrifugation is commonly employed to remove insoluble cellular debris from the protein-containing supernatant. A common disadvantage of these protocols is the biased selection of certain protein classes occurring during the course of the lysate preparation. In addition, almost all of the techniques currently used in proteomics combine a variety of fractionation and separation steps before analysis by MS. These protocols include purifications that yield a specific group of compounds for analysis rather than measurement of the cell in entirety. Typical separation procedures include affinity separation methods [44–

Published online February 17, 2006

Address reprint requests to Professor L. M. Smith, Department of Chemistry, University of Wisconsin-Madison, 1101 University Avenue, Madison, WI 53706-1396, USA. E-mail: smith@chem.wisc.edu

48], 1-D or 2-D gel electrophoresis [49–52], and 1-D or 2-D chromatographic separations [53–56]. These methods have high-resolution and can provide a great quantity of information. However, the limitation of these methods is their laborious and time-consuming nature.

Many methods and technologies have been developed and applied in single cell analysis. Generally, a single cell has been isolated using micropipettes, optical traps, or dielectrophoresis [57–61]. After injection into a capillary or into a microfluidic channel, the intact cell undergoes lysis to release cellular components. Capillary electrophoresis has commonly been used as a separation technique before MS analysis [62–64]. However, the sensitivity of current mass spectrometers is not great enough to detect many components in the ultratrace amounts present in a single cell. Consequently, the number of proteins identifiable by current single cell methods is limited. MALDI-TOF mass spectrometry has been shown to be effective for peptide profiling directly from a single cell [65–67]. In this approach, a single cell is transferred onto a MALDI plate and 2,5-dihydroxybenzoic acid (DHB) matrix solution is used as a cell rinsing reagent in addition to acting as the MALDI matrix. Both extracellular and intracellular salts can be removed in a stepwise fashion by spot-to-spot cell transfers to a fresh matrix drop on the sample plate.

In this report, we present a simple and direct approach to obtain unique fingerprint information to differentiate mammalian cell types using MALDI-MS. The approach is rapid and works for a few cells (~1000), and as such is potentially well suited to high-throughput analyses. Cells were placed in matrix solution and analyzed by MALDI-MS directly after the mixing of cell contents with the matrix solution. Three different MALDI matrices (DHB, SA, and CHCA) were used to directly extract and analyze mammalian cells and the MALDI-TOF results are compared among these matrices. Three different mammalian cell lines were analyzed using DHB as the extraction matrix, and found to produce unique and reproducible MALDI fingerprints that can be utilized to identify these cell lines. MALDI-PSD, MALDI TOF-TOF, and MALDI-FTICR direct analyses of these samples showed limited ability to produce useful sequence information. LC-ESI-MS/MS analyses of a tryptic digestion of these samples did provide useful sequence information, permitting identification of a number of proteins from the different cell lines.

Experimental

Reagents

The cell lines K562, BHK21, and GM15226 were all obtained from ATCC (Manassas, VA). K562 and GM15226 were cultured in 5% CO₂ in a 37 °C incubator with RPMI-1640 containing 1% glutamine, 1% penicillin-streptomycin, and 15% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) as culture medium.

BHK21 was cultured in Dulbecco's modified essential medium/F-12 medium supplemented with 5% FBS and penicillin-streptomycin (60 µg/mL) and was incubated in 5% CO₂ in a 37 °C incubator.

DHB (2,5-dihydroxybenzoic acid), 4-hydroxy- α -cyanocinnamic acid (4-CHCA), 3,5-dimethyl-4-hydroxycinnamic acid (sinapinic acid), and a Proteo-Mass Peptide and Protein MALDI-MS Calibration Kit were obtained from Sigma (St. Louis, MO). Sequencing-grade modified trypsin was from Promega (Madison, WI). HPLC-grade water was obtained from Honeywell B&J (Muskegon, MI). HPLC-grade acetonitrile and formic acid were obtained from Fischer Scientific (Philadelphia, PA).

Sample Preparation

DHB extraction and rinsing of cells. Approximately 10⁶ cells taken directly from cell culture were centrifuged at 1600 rpm for 5 min at 5 °C, and the supernatant was discarded. The cell pellet was either resuspended directly in 100 µL MALDI matrix solution (only in the case of the “no rinse” comparison in Figure 5) or subsequently washed twice with 100 µL 10 mg/mL DHB solution before resuspension in the same solution (for all other analyses). The washing process involved vortexing the samples for a few seconds, centrifuging the samples, and discarding the supernatant. After the second wash step, the cells were resuspended in DHB solution and analyzed by MALDI mass spectrometry.

Tryptic Digestion for LC-ESI-MS/MS analysis. One mL of the DHB extracted supernatant was dried in a SpeedVac (Thermo Savant, Holbrook, NY) and a standard in-solution tryptic digestion procedure was performed. Briefly, the sample was reconstituted in 200 µL 8M urea, 0.4M NH₄HCO₃, reduced by adding 10 µL 100 mM dithiothreitol in 50 mM NH₄HCO₃ and then alkylated by the addition of 20 µL 100 mM iodoacetamide in 50 mM NH₄HCO₃. The urea concentration was diluted to <2 M by the addition of 770 µL water, followed by the addition of 40 µL 0.25 µg/µL modified trypsin (Promega, Madison, WI). Tryptic digestion was carried out overnight at 37 °C. The digested samples were purified using C18 ziptips (Millipore, Bedford, MA) and then resuspended in 10 µL 50% ACN + 0.1% formic acid before LC-ESI-MS/MS analysis.

MALDI-TOF MS analysis. MALDI-TOF mass spectra of DHB rinsed and resuspended cells were obtained using a Voyager-DE STR mass spectrometer (Applied Biosystems, Foster City, CA). One µL of the DHB rinsed and resuspended cells were spotted directly onto the MALDI plate (Figures 1, 4, 5, 6) or mixed with 1 µL matrix (4-CHCA or sinapinic acid, Figure 3). A pulsed nitrogen laser (337 nm) was used for desorption/ionization and positive ion mass spectra were acquired in linear mode. The total acceleration voltage was 25 kV, grid voltage at 92.7%, guide wire voltage at 0.25%,

and a delay time of 250 ns was used. External mass calibration was performed using insulin and angiotensin I. Spectra were obtained by accumulating data from 50 laser shots.

Photomicrographs of MALDI samples were obtained using a Nikon Eclipse ME600 microscope (Melville, NY).

MALDI-TOF/TOF. Samples of the DHB rinsed and resuspended cells were sent to Bruker and Applied Biosystem for MALDI-TOF/TOF analysis on a Bruker UltraflexII or an ABI 4700, respectively. In each case, 1 μ L of DHB rinsed and resuspended cells were directly spotted onto the MALDI plate for analysis, or alternatively mixed 1:1 with α -cyano-3-hydroxycinnamic acid matrix (5 mg/mL in 50% ACN + 0.1% TFA), followed by spotting onto the MALDI plate.

Samples were analyzed in positive ion mode in both reflector and MS/MS acquisition modes with a laser repetition rate of 200 Hz. In MS/MS mode, 1 kV collision energy (with CID gas ON) was used to fragment the peptides. In both reflector and MSMS modes, the instrument default calibration was used.

MALDI-FTICR analysis. The tryptic digest of the DHB extracted supernatant was analyzed by MALDI-FTICR. One μ L of sample was mixed 1:1 with DHB matrix and spotted onto a MALDI plate. MALDI-FTICR experiments were performed on an Ionspec Fourier transform mass spectrometer (Lake Forest, CA) equipped with a 7.0-T actively shielded superconducting magnet. An external 337 nm nitrogen laser (Laser Science, Inc., Franklin, MA) was used for ionization/desorption. A quadrupole ion guide was used to transfer the ions to the ICR cell, which was differentially pumped.

MALDI-PSD fragmentation analysis. The tryptic digest of the DHB extracted supernatant was analyzed by MALDI-PSD. One μ L of sample was mixed 1:1 with DHB matrix and spotted onto a MALDI plate. PSD spectra were obtained using a Voyager-DE STR mass spectrometer (Applied Biosystems). By using a timed ion selector, different precursor ions can be selected and subjected to fragmentation. To obtain a complete PSD spectrum, a series of reflectron TOF spectral segments were acquired, each optimized to focus fragment ions within different m/z ranges. A composite PSD spectrum was generated using the Voyager 5.1 Dataexplore software (Applied Biosystem).

LC-ESI-MS/MS analysis. The same tryptic digest of the DHB extracted supernatant was also analyzed by LC-ESI-MS/MS. The LC-ESI-MS/MS system consists of an HPLC connected to an ESI ion trap mass spectrometer (Surveyor HPLC and LCQ deca XPplus, Thermo Finnigan, San Jose, CA). A fritless 100 $\times$ 365 μ m fused-silica capillary micro-column was prepared by pulling the tip of the capillary to $\sim$ 2 μ m with a P-2000 laser puller (Sutter Instruments Co., Novato, CA), and packing with

10 cm of 5 μ m diameter, 300 Å pore size C18 beads (Western Analytical Products, Inc., Murrieta, CA). The capillary column was connected to the HPLC through a polyetheretherketone (PEEK) microcross with a platinum wire inserted into the flow-through to supply a spray voltage of 1.8 kV.

The lyophilized, zip-tip purified tryptic digest was reconstituted in 10 μ L of 0.1% formic acid in water. After injecting 10 μ L of sample onto the column, a 30 min load/wash with 95% buffer A (0.1% vol/vol formic acid in water) was applied at a flow-rate of 1 μ L/min. Peptides were eluted using a linear gradient of 5% buffer B (0.1% vol/vol formic acid in acetonitrile) to 80% buffer B over 160 min with a constant flow rate of 300 nL/min.

The ion trap mass spectrometer was operated in "biggest 3" mode. Each full-mass scan was performed between 400–2000 m/z , followed by three MS/MS scans of the three highest intensity parent ions at 45% relative collision energy. Dynamic exclusion was enabled with a repeat count of 3, exclusion duration of 3 min, and repeat duration of 1min.

The acquired MS/MS spectra were searched against either a human (K562 and GM15226) or a rodent (BHK21) protein database (created from the non-redundant protein database available through NCBI) using the SEQUEST program (Thermo Finnigan, San Jose, CA). Peptide matches were accepted if they met the following criteria: (1) fully tryptic; (2) $\Delta C_n < 0.1$; (3) Xcorr scores > 1.9 for +1 peptides, 2.2 for +2 peptides, and 3.75 for +3 peptides.

Results and Discussion

To optimize the direct MALDI-MS analyses of cells suspended directly in matrix, K562 was selected as a model cell line because of its robustness and relative stability. K562 is a human myelo-monocytic cell line that was derived from a chronic myelogenous leukemic patient [68].

After centrifugation and removal of the cell growth medium, the cell pellet was placed in a 10 mg/mL 2,5-dihydroxybenzoic acid (DHB) solution to allow mixing of the cell contents with the matrix solution. This mixture of cells and DHB matrix was analyzed directly by MALDI-MS without further purification. It was somewhat surprising that unique and reproducible mass spectra were obtained without further sample clean-up. The mass spectra of whole cell mixtures generally exhibit a large number of discrete peaks in the m/z range between 1000 and 20,000 Da. Three spectra of K562 cell mixtures from independent cell cultures are shown in Figure 1.

Replicate experiments were performed on three successive generations and over 20 discontinuous generations of K562 cells and the same peak pattern as shown in Figure 1 was obtained each time. This result indicates that despite expected variations in some protein expression levels due to growth condi-

Figure 1. MALDI-TOF mass spectra of K562 cells in DHB matrix from three independent cell cultures.

tions and non-synchronized cell growth cycles, this cell line yields a distinct mass spectral signature.

The same cell mixture was also analyzed using SDS-PAGE (data not shown). An intense band around 66 kDa corresponds to the molecular weight of fetal bovine serum albumin (fBSA), which is a main component in the culture medium. Several less intense bands in the 30–60 kDa mass range were also observed. As shown in the mass spectrum of this same cell mixture (Figure 1), only ions of 22 kDa or lower m/z were detected with high intensity. Although this sample was analyzed using various settings of MALDI-MS instrumental parameters favoring the detection of larger proteins, the only peak observed above 22 kDa was a broad and low-intensity peak at 66 kDa corresponding to fBSA (data not shown). Larger proteins are often not detected by MALDI analysis because of a combination of widely variable ionization efficiencies and the lower detection efficiency of the instrument for higher mass ions [69]. In this case, there is possibly ionization suppression and/or a smaller population of desorbed larger proteins due to the presence of so many smaller proteins and peptides.

Although MALDI is relatively tolerant of impurities, the ability to obtain quality mass spectra is highly dependent on crystal formation, salt concentration, and sample size. As observed during the course of these experiments, only cells embedded in DHB matrix crystals yielded highly intense signals. Figure 2 shows an example of cells crystallized with DHB on a MALDI sample plate observed under a microscope.

Effect of Different Matrix

Careful selection of the appropriate matrix is an important issue for optimum MALDI analyses. 4-hydroxy- α -cyanocinnamic acid (4-CHCA) and 3,5-dimethyl-4-hydroxycinnamic acid (SA) have both previ-

Figure 2. K562 cells co-crystallized with DHB matrix.

ously been used as matrices for bacterial cell analyses [3, 4, 7]. DHB has been the most commonly used matrix for single-cell MALDI studies because it serves to aid in microdissections by stabilizing cell membranes and also best eliminates the salt effect [67, 70]. To determine the optimum matrix for mammalian whole-cell analyses, each of these three MALDI matrix solutions was applied to the centrifuged K562 cell pellet and the resulting MALDI spectra are shown in Figure 3. The best overall results were obtained using pure DHB as matrix, and accordingly it was employed for all the subsequent MALDI analyses.

Analysis of Supernatant and Effect of Rinsing Steps

There is a possibility that some proteins are lost in the supernatant if indeed the cells lyse in matrix solution. Therefore, we analyzed the supernatant after the initial suspension and subsequent centrifugation of the cells in DHB solution. After cell culture medium removal, the

Figure 3. MALDI-TOF mass spectra of K562 cells extracted in different matrices.

cell pellet was resuspended in DHB solution and centrifuged again. The supernatant was reserved for MALDI analysis, labeled supernatant 1 (Figure 4a). After resuspending and centrifuging a second time in DHB, the supernatant was again reserved for MALDI analysis (Figure 4b, supernatant 2). Finally, the cells were resuspended a third time in DHB and an aliquot including cells was analyzed by MALDI-MS (Figure 4c, cells). The MALDI spectrum of the supernatant from the first DHB rinse (Figure 4a) shows a single, high intensity peak at 4962 m/z . Figure 4b, corresponding to the supernatant from the second DHB rinse, shows a series of peaks throughout the mass range of 1000 to 15,000 kDa, which are similar although less intense than those from analysis of cells (Figure 4c). The similarity between the peak pattern evident in Figure 4b and c shows that likely the cells are being lysed in DHB solution and cytoplasmic proteins are released into the supernatant.

Interestingly, the peak at 4962 Da completely disappears with the second DHB rinse step. This result shows that a large amount of protein with m/z 4962 Da was extracted into the matrix solution during the first DHB rinse step. A previous study of human erythroleukemia cells [41] identified a peak at m/z 4962 as thymosin β 4, a 43 amino acid polypeptide rich in glutamic acid and lysine. Thymosin β 4 was shown to be widely distributed in many tissues and is thought to be an important mediator in cell proliferation, migration, and differentiation.

Rinsing in DHB matrix has been shown to remove salts from MALDI samples and also probably lyses the cells [3, 4, 7] releasing intracellular proteins. For comparison, a portion of BHK21 cells were also MALDI analyzed without any rinsing steps. The results are

Figure 4. Representative MALDI mass spectra of K562 cells extracted and rinsed in DHB matrix. (a) Supernatant from the first DHB rinse; (b) supernatant from the second DHB rinse; (c) K562 cells after two DHB rinses.

Figure 5. MALDI-TOF mass spectra of BHK21 cells. (a) No rinse; (b) DHB matrix solution rinse (see Experimental section for details). The inset is the full scale spectrum of (a).

shown in Figure 5, with both spectra plotted on the same intensity scale. The main difference between the spectra is the intensity of the peak at 4962 Da. Without rinsing (Figure 5a), the 4962 Da peak is much more intense than in the spectrum of cells rinsed in DHB solution (Figure 5b). This result again shows that this protein was extracted into the matrix solution during the DHB rinsing step. Comparing Figure 5a and b shows that the peaks at higher m/z possess both higher intensity and better S/N when the DHB rinsing step is included.

A series of peaks centered around 11 and 14 kDa do not appear intensely in the spectra obtained from cells without DHB rinsing. These two groups of peaks have been determined by LC-ESI-MS/MS to correspond to different forms of histones (see below). Histones are a common protein in all eukaryotic cells and are responsible for packaging and ordering the DNA into nucleosomes. After DHB matrix rinsing, the intensities of the histone peaks increase significantly, probably because the cells were lysed and the histones were released from the nuclear membrane. Peaks at m/z 11,290, 11,323, and 11,432 Da are associated with multiply modified forms of histone H4. The peak at 13,763 is identified as histone H2B*. A grouping of three peaks at 13,993, 14,022, and 14,123 Da correspond to the different forms of histone H2A*.

MALDI Analysis of Three Mammalian Cell Lines

Two additional mammalian cell lines, GM15226 and BHK21, were analyzed following the optimized DHB suspension and rinsing procedure presented above. BHK21 is a fibroblast cell line derived from baby hamster kidney [71], and GM15226 is a human lymphoblast cell line. The results obtained from whole cell MALDI-MS analysis of the three different cell lines are shown in Figure 6. As shown in Figure 6, profiles of the three different cell types are easily distinguished by

Figure 6. MALDI-TOF mass spectra of three different mammalian cell lines in DHB. The asterisk denotes peaks in each MALDI spectrum that are unique to that cell line.

visual inspection. While many of the major peaks observed for all three cell lines are common, there are also a number of unique peaks distinguishing each cell line. Both the peaks in common and the peaks specific to a cell line observed during the MALDI-MS analysis of these three cell lines are summarized in Table 1. The specific peaks for each variety of cell can be utilized as a “fingerprint” to identify that cell type.

Attempts to Identify Proteins—Peptide Mass Fingerprint, PSD, TOF-TOF, MALDI-FTICR, LC-ESI-MS/MS

MALDI-MS is a rapid and straightforward method to generate peak patterns from whole cells. The analyses of three different cell lines shown here produce unique patterns, and it is desirable and important to identify those peaks specific to each different cell line. This problem of protein/peptide identification of a specific peak in a complicated MALDI spectrum is a challenge that is commonly faced by many researchers in the field of biomarker analyses [20, 22–24]. Here, we explored both bottom-up and top-down approaches to identify

some of the proteins present in the DHB extractant of whole cells.

A standard reduction and alkylation followed by tryptic digestion was performed on the supernatant of K562 cells in DHB matrix solution (see the Experimental section for details). The digested mixture was analyzed by LC-ESI-MS/MS (see below) and by MALDI-MS for peptide mass fingerprint identification of proteins (data not shown). Several peaks identified as peptides originating from histones (1777, 1761, 1797, 1326, 1311, 1094) and ribosomal proteins (1838, 1233, 942, 1087) in the LC-ESI-MS/MS run of this same sample were also evident in the MALDI-TOF spectra. However, due to the extreme complexity of this sample, MALDI mass fingerprinting was not attempted for identification of the proteins.

The same digested mixture used for MALDI peptide mass fingerprinting was also analyzed by MALDI-post source decay (PSD). PSD is a fragmentation method used in MALDI-MS analyses to obtain additional information for identification. The ions fragmenting in the field-free region of the drift tube (metastable decay) are separated by their difference in kinetic energy using an ion reflector. However, PSD is not very sensitive and cannot handle very complex samples [66, 67]. In addition, PSD has an effective upper m/z limit of 2000 Da because peptides larger than 2000 Da tend not to fragment very well [66, 67]. We attempted PSD fragmentation of four different peaks below 2000 Da [1326, 1233, 1094, 942] present in the MALDI spectrum of K562 cells. We found that the signal intensity was too low and not enough fragments could be generated for confident identification.

MALDI-FTICR is well known for high sensitivity and high-resolution. The same tryptically digested mixture was analyzed by MALDI-FTICR. Three peptides with the highest signal intensities (2212, 1795, 1695 m/z) were subjected to fragmentation analysis in an attempt to identify them. Stored waveform inverse Fourier transform (SWIFT) technique [72–75] was employed for mass-selective ion injection, accumulation, and isolation, followed by sustained off resonance irradiation and collision-induced dissociation (SORI-CID) [76–78] to fragment selected peptide ions. Nitrogen gas was used to cool the ions and dissociate the peptides. The SORI procedure (1000 ms in duration) employed a frequency offset ranging from 1000 to 600 Hz and

Table 1. Comparison of peaks in MALDI-MS analysis of three different mammalian cell types

Cell type	Common markers among all three cell lines (m/z)	Common markers between two cell lines (m/z)	Unique cell markers (m/z)
K562	3005, 3156, 3457, 4963, 6273, 6879, 8552, 11290, 13762	1264, 1525, 5730, 7873	1802, 1876, 1887, 2002, 5648, 6322, 6996
GM15226	3005, 3156, 3458, 4962, 6275, 6880, 8553, 11289, 13763	1264, 1526, 5605, 5735, 10785	1675, 5443, 6665, 7813, 9109
BHK21	3004, 3154, 3456, 4962, 6273, 6879, 8552, 11292, 13760	5601, 7872, 10775	1568, 2229, 3789, 6216, 6640, 9278, 10020, 10128

Table 2. Proteins identified from DHB extraction of K562 cell by LC/MS/MS

Proteins identified Histones	Peptide sequences
H2BL_HUMAN Histone H2B.1 (H2B/1)	AMGIMNSFVNDIFER, AM#GIMNSFVNDIFER, AMGIM#NSFVNDIFER
H1 histone family, member 1	ALAAAGYDVEK
H1 histone family, member 5	ALAAGGYDVEK
Histone H4 homologue	DNIQGITKPAIR
	ISGLIYEETR
H1 histone family, member 3	SGVSLAALKK, KASGPPVSELITK
Histone H2A	VGAGAPVYLAHVLEYLTAEILELAGNAAR
Ribosomal proteins	
Ribosomal protein L13	STESLQANVQR
Ribosomal protein L8	AVVGWVAGGGR
Ribosomal protein L29	AQAAAPASVPAQAPK
Ribosomal protein S19	DVNQQEFVR, AJLAAFLK
Ribosomal protein L26	KDNEVQVVR
30S ribosomal protein S1	VKHPSDIISIGDEISVK
Others	
S34426 nuclease sensitive element-binding protein 1	GAEAAANVTGPGGVPVQGSK
	SVGDGETVEFDVVEGEK
Homo sapiens ATP synthase	QTSGGPVDASSEYQQELER
^a MARCKS-like protein (myristoylated alanine-rich protein kinase C substrate)	GDVTAEAAAGASPAK
^a Stathmin 1	ASGOAFELILSPR
^a Cytokine induced protein 29kDa	FGIVTSSAGTGTTEDEAK
Homo sapiens cytochrome C, somatic	TGQAPGYSYTAANK
RPS21 protein	MGESDDSILR
Hypothetical protein	GGGCVWEAEAGGSR
Nascent-polypeptide-associated complex alpha polypeptide	IEDLSQQAQLAAAEK
RICKEN cDNA 2210402A09	AEAGAGSATEFQFR
Alpha NAC/1.9.2 protein	NEDLSQQAQLAAAEK
Human thioredoxin mutant with Cys 73 replaced by Ser	TAFQEALDAAGDK
Chaperonin 10-related protein	VLQATVVAVGSGSK
Pheromone biosynthesis activating neuropeptide	TCLLRQLTRK
Cytokeratin 9	VQALEEANNNDLENK
Keratin 1	FLEQQNQVLQTK, SLDLDSIIAEVK
Keratin 10, type I, epidermal	ALEESNYELEGK, SLLEGE GSSGGGGR

^aProteins specific to K562 leukemia cell or human cancer cells

amplitudes of 4.0–5.0 V (base to peak), depending on the desired level of fragmentation and the energy needed to dissociate the peptide. Because SORI-CID is a relatively low-energy fragmentation process, it is generally most effective for peptides with molecular weight below 2000 Da. As for the PSD experiments, too few fragment ions were observed to allow confident identification of the three peptides. It should be noted that newer fragmentation processes introduced over the last several years, such as electron capture dissociation (ECD) [79–82] and electron-transfer dissociation (ETD) [83, 84], have significantly increased the ability of FT instruments to obtain high-quality fragmentation results.

MALDI-TOF/TOF instruments are currently used not only for the acquisition of peptide mass fingerprint spectra, but also for subsequent protein identification through CID fragmentation of selected precursor ions. MALDI-TOF/TOF instruments have also recently been employed for top-down proteomic measurements, with

one study reporting fragmentation of thioredoxin, an 11 kDa protein [85]. However, the upper *m/z* limit for fragmentation is generally reported as between 4000–5000 Da.

A sample of K562 cells extracted in DHB (see Figure 1) was analyzed on two different MALDI-TOF/TOF instruments (Bruker UltraflexII and ABI 4700). This sample represents a top-down approach since the proteins have not been digested and are presumably intact. Similar results were obtained with both TOF-TOF instruments. A total of three peptides were confidently identified by CID fragmentation in both instruments. The identified peptides were 1366.74 Da (Histone H4-1), 1800.07 Da (Histone H2A.1), and 1871.11 Da (Histone H2A.5). It is interesting to note that no peptide above 2000 Da was identified even though there are several high intensity peaks around 3000 Da. Although instrument manufacturer specifications claim that peptides in the 4000–5000 Da range may be utilized for CID fragmen-

tation analysis, this result suggests that the effective upper mass limit for unknown peptides in a complex mixture remains around 2000 Da.

LC-ESI-MS/MS Analysis

The same digestion solution analyzed by MALDI-TOF, MALDI-PSD, and MALDI-FTICR was separated and analyzed using LC-ESI-MS/MS on an ion trap mass spectrometer. Because the complicated peptide mixture was separated over an LC column before MS/MS analysis, significantly more peptides and proteins were identified. The data were searched against a human protein database using the Sequest database searching program [86]. The combined database search results from two separate LC-ESI-MS/MS runs of the same digestion solution are shown in Table 2. Histones and ribosomal proteins are the two main groups of proteins identified. These results are consistent with those observed in MALDI peptide mass fingerprint analysis of the same sample, with several of the identified peptide molecular weights matching peaks observed in the MALDI spectra. Several different forms of histone appear to be highly abundant in the DHB extraction solution. Several other proteins found in leukemia or human tumor cells, such as MARCKS-like protein, Stathmin 1, and Cytokine-induced protein were also identified.

Conclusions

A simple, direct, rapid, and accurate mass spectrometric method has been developed for identification of different cell types. Cellular proteins were released by DHB extraction of intact cells and analyzed by MALDI mass spectrometry. The entire analytical process could be completed within 10 min. Three different types of mammalian cells were investigated and found to yield unique and reproducible MALDI-MS patterns, which can be used as a fingerprint to identify and distinguish these different cell-lines. Attempts to identify these unique biomarker peaks using several different MALDI top-down technologies (TOF-TOF, PSD, FTICR) were largely unsuccessful. Using the bottom-up proteomics approach of LC-ESI-MS/MS analysis of a tryptic digestion of the DHB extracted proteins, many proteins were identified. Future developments in direct MALDI top-down technology should allow for direct identification of these biomarker peaks without prior separation. This potentially powerful approach is well suited for automation and high throughput due to its simplicity.

Acknowledgments

Supported by NIH/NHLBI grant N01-HV-28,182. The authors thank Kimberly Kutz, Dr. Lingjun Li, and Gary Girdaukas (School of Pharmacy, UW-Madison) for help with FTICR-MS experiments. They also thank the UW-Madison Keck Center for cell culturing facilities, and Dr. Robert J. Hamers for microscope facility.

References

- Karas, M.; Bachmann, D.; Bahr, U.; Hillenkamp, F. Matrix-Assisted Ultraviolet-Laser Desorption of Nonvolatile Compounds. *Int. J. Mass Spectrom. Ion Processes* **1987**, *78*, 53–68.
- Karas, M.; Hillenkamp, F. Laser Desorption Ionization of Proteins with Molecular Masses Exceeding 10,000 Daltons. *Anal. Chem.* **1988**, *60*(20), 2299–2301.
- Krishnamurthy, T.; Ross, P. L. Rapid identification of bacteria by direct matrix-assisted laser desorption/ionization mass spectrometric analysis of whole cells. *Rapid Commun. Mass Spectrom.* **1996**, *10*(15), 1992–1996.
- Krishnamurthy, T.; Rajamani, U.; Ross, P. L.; Janhour, R.; Nair, H.; Eng, J.; Yates, J.; Davis, M. T.; Stahl, D. C.; Lee, T. D. Mass spectral investigations on microorganisms. *J. Toxicol. Toxin Rev.* **2000**, *19*(1), 95–117.
- Warscheid, B.; Fenselau, C. A targeted proteomics approach to the rapid identification of bacterial cell mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *Proteomics* **2004**, *4*(10), 2877–2892.
- English, R. D.; Warscheid, B.; Fenselau, C.; Cotter, R. J. Bacillus spore identification via proteolytic peptide mapping with a miniaturized MALDI TOF mass spectrometer. *Anal. Chem.* **2003**, *75*(24), 6886–6893.
- Krishnamurthy, T.; Ross, P. L.; Rajamani, U. Detection of pathogenic and non-pathogenic bacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **1996**, *10*(8), 883–888.
- Vaidyanathan, S.; Winder, C. L.; Wade, S. C.; Kell, D. B.; Goodacre, R. Sample preparation in matrix-assisted laser desorption ionization mass spectrometry of whole bacterial cells and the detection of high mass (> 20 kDa) proteins. *Rapid Commun. Mass Spectrom.* **2002**, *16*(13), 1276–1286.
- Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **1999**, *17*(10), 994–999.
- Smolka, M. B.; Zhou, H. L.; Purkayastha, S.; Aebersold, R. Optimization of the isotope-coded affinity tag-labeling procedure for quantitative proteome analysis. *Anal. Biochem.* **2001**, *297*(1), 25–31.
- Gygi, S. P.; Rist, B.; Griffin, T. J.; Eng, J.; Aebersold, R. Proteome analysis of low-abundance proteins using multidimensional chromatography and isotope-coded affinity tags. *J. Proteome Res.* **2002**, *1*(1), 47–54.
- Han, D. K.; Eng, J.; Zhou, H. L.; Aebersold, R. Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry. *Nat. Biotechnol.* **2001**, *19*(10), 946–951.
- Thomson, S. T.; Il; Figeys, D. O-18 Labeling: A tool for proteomics. *Rapid Commun. Mass Spectrom.* **2001**, *15*(24), 2456–2465.
- Yao, X. D.; Freas, A.; Ramirez, J.; Demirev, P. A.; Fenselau, C. Proteolytic O-18 labeling for comparative proteomics: Model studies with two serotypes of adenovirus. *Anal. Chem.* **2001**, *73*(13), 2836–2842.
- Mirgorodskaya, O. A.; Kozmin, Y. P.; Titov, M. I.; Korner, R.; Sonksen, C. P.; Roepstorff, P. Quantitation of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry using O-18-labeled internal standards. *Rapid Commun. Mass Spectrom.* **2000**, *14*(14), 1226–1232.
- Hunt, A. N.; Clark, G. T.; Neale, J. R.; Postle, A. D. A comparison of the molecular specificities of whole cell and endonuclear phosphatidylcholine synthesis. *FEBS Lett.* **2002**, *530*(1/3), 89–93.
- Washburn, M. P.; Ulaszek, R.; Deciu, C.; Schieltz, D. M.; Yates, J. R. Analysis of quantitative proteomic data generated via multidimensional protein identification technology. *Anal. Chem.* **2002**, *74*(7), 1650–1657.
- Oda, Y.; Huang, K.; Cross, F. R.; Cowburn, D.; Chait, B. T. Accurate quantitation of protein expression and site-specific phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America*; **1999**; pp 6591–6596.
- Goshe, M. B.; Smith, R. D. Stable isotope-coded proteomic mass spectrometry. *Curr. Opin. Biotechnol.* **2003**, *14*(1), 101–109.
- Wright, G. L.; Cazares, L. H.; Leung, S. M.; Nasim, S.; Adam, B. L.; Yip, T. T.; Schellhammer, P. F.; Gong, L.; Vlahou, A. Proteinchip(R) surface enhanced laser desorption/ionization (SELDI) mass spectrometry: A novel protein biochip technology for detection of prostate cancer biomarkers in complex protein mixtures. *Prostate Cancer Prostatic Dis.* **1999**, *2*(5/6), 264–276.
- Reyzer, M. L.; Caldwell, R. L.; Dugger, T. C.; Forbes, J. T.; Ritter, C. A.; Guix, M.; Arteaga, C. L.; Caprioli, R. M. Early changes in protein expression detected by mass spectrometry predict tumor response to molecular therapeutics. *Cancer Res.* **2004**, *64*(24), 9093–9100.
- Wiesner, A. Detection of tumor markers with ProteinChip(R) technology. *Curr. Pharmaceut. Biotechnol.* **2004**, *5*(1), 45–67.
- Sun, Z. H.; Fu, X. L.; Zang, L.; Yang, X. L.; Liu, F. Z.; Hu, G. X. A protein chip system for parallel analysis of multi-tumor markers and its application in cancer detection. *Anticancer Res.* **2004**, *24*(2C), 1159–1165.
- Shiwa, M.; Nishimura, Y.; Wakatabe, R.; Fukawa, A.; Arikuni, H.; Ota, H.; Kato, Y.; Yamori, T. Rapid discovery and identification of a tissue-specific tumor biomarker from 39 human cancer cell lines using the SELDI ProteinChip platform. *Biochem. Biophys. Res. Commun.* **2003**, *309*(1), 18–25.
- Nielsen, P. A.; Olsen, J. V.; Podtelejnikov, A. V.; Andersen, J. R.; Mann, M.; Wisniewski, J. R. Proteomic mapping of brain plasma membrane proteins. *Mol. Cell. Proteom.* **2005**, *4*(4), 402–408.

26. Gruhler, A.; Olsen, J. V.; Mohammed, S.; Mortensen, P.; Faergeman, N. J.; Mann, M.; Jensen, O. N. Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. *Mol. Cell. Proteom.* **2005**, *4*(3), 310–327.
27. Cantin, G. T.; Yates, J. R. Strategies for shotgun identification of post-translational modifications by mass spectrometry. *J. Chromatogr. A* **2004**, *1053*(1/2), 7–14.
28. Chang, E. J.; Archambault, V.; McLachlin, D. T.; Krutchinsky, A. N.; Chait, B. T. Analysis of protein phosphorylation by hypothesis-driven multiple-stage mass spectrometry. *Anal. Chem.* **2004**, *76*(15), 4472–4483.
29. Peng, J. M.; Schwartz, D.; Elias, J. E.; Thoreen, C. C.; Cheng, D. M.; Marsischky, G.; Roelofs, J.; Finley, D.; Gygi, S. P. A proteomics approach to understanding protein ubiquitination. *Nat. Biotechnol.* **2003**, *21*(8), 921–926.
30. Denison, C.; Rudner, A. D.; Gerber, S. A.; Bakalarski, C. E.; Moazed, D.; Gygi, S. P. A proteomic strategy for gaining insights into protein sumoylation in yeast. *Mol. Cell. Proteom.* **2005**, *4*(3), 246–254.
31. Wright, M. E.; Han, D. K.; Aebersold, R. Mass spectrometry-based expression profiling of clinical prostate cancer. *Mol. Cell. Proteom.* **2005**, *4*(4), 545–554.
32. Denison, C.; Kirkpatrick, D. S.; Gygi, S. P. Proteomic insights into ubiquitin and ubiquitin-like proteins. *Curr. Opin. Chem. Biol.* **2005**, *9*(1), 69–75.
33. Mann, M.; Jensen, O. N. Proteomic analysis of post-translational modifications. *Nat. Biotechnol.* **2003**, *21*(3), 255–261.
34. Mann, M.; Ong, S. E.; Gronborg, M.; Steen, H.; Jensen, O. N.; Pandey, A. Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. *Trends Biotechnol.* **2002**, *20*(6), 261–268.
35. McLachlin, D. T.; Chait, B. T. Analysis of phosphorylated proteins and peptides by mass spectrometry. *Curr. Opin. Chem. Biol.* **2001**, *5*(5), 591–602.
36. Gygi, S. P.; Rist, B.; Aebersold, R. Measuring gene expression by quantitative proteome analysis. *Curr. Opin. Biotechnol.* **2000**, *11*(4), 396–401.
37. Kuster, B.; Mann, M. Identifying proteins and post-translational modifications by mass spectrometry. *Curr. Opin. Struct. Biol.* **1998**, *8*(3), 393–400.
38. Hixson, K. K.; Rodriguez, N.; Camp, D. G.; Strittmatter, E. F.; Lipton, M. S.; Smith, R. D. Evaluation of enzymatic digestion and liquid chromatography-mass spectrometry peptide mapping of the integral membrane protein bacteriorhodopsin. *Electrophoresis* **2002**, *23*(18), 3224–3232.
39. Blonder, J.; Goshe, M. B.; Moore, R. J.; Pasa-Tolic, L.; Masselon, C. D.; Lipton, M. S.; Smith, R. D. Enrichment of integral membrane proteins for proteomic analysis using liquid chromatography-tandem mass spectrometry. *J. Proteome Res.* **2002**, *1*(4), 351–360.
40. Wu, C. C.; MacCoss, M. J.; Howell, K. E.; Yates, J. R. A method for the comprehensive proteomic analysis of membrane proteins. *Nat. Biotechnol.* **2003**, *21*(5), 532–538.
41. Chen, Y. J.; Wall, D. Lubman, D. M. Rapid identification and screening of proteins from whole cell lysates of human erythroleukemia cells in the liquid phase, using non-porous reversed phase high-performance liquid chromatography separations of proteins followed by multi-assisted laser desorption/ionization mass spectrometry analysis and sequence database searching. *Rapid Commun. Mass Spectrom.* **1998**, *12*(24), 1994–2003.
42. van Adrichem, J. H. M.; Bornsen, K. O.; Conzelmann, H.; Gass, M. A. S.; Eppenberger, H.; Kresbach, G. M.; Ehrat, M.; Leist, C. H. Investigation of protein patterns in mammalian cells and culture supernatants by matrix assisted laser desorption/ionization mass spectrometry. *Anal. Chem.* **1998**, *70*(5), 923–930.
43. Shetty, J.; Diekmann, A. B.; Jayes, F. C. L.; Sherman, N. E.; Naaby-Hansen, S.; Flickinger, C. J.; Herr, J. C. Differential extraction and enrichment of human sperm surface proteins in a proteome: Identification of immunocontraceptive candidates. *Electrophoresis* **2001**, *22*(14), 3053–3066.
44. Ji, J. Y.; Chakraborty, A.; Geng, M.; Zhang, X.; Amini, A.; Bina, M.; Regnier, F. Strategy for qualitative and quantitative analysis in proteomics based on signature peptides. *J. Chromatogr. B* **2000**, *745*(1), 197–210.
45. Amini, A.; Chakraborty, A.; Regnier, F. E. Simplification of complex tryptic digests for capillary electrophoresis by affinity selection of histidine-containing peptides with immobilized metal ion affinity chromatography. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2002**, *772*(1), 35–44.
46. Salomon, A. R.; Ficarro, S. B.; Brill, L. M.; Brinker, A.; Phung, Q. T.; Ericson, C.; Sauer, K.; Brock, A.; Horn, D. M.; Schultz, P. G.; Peters, E. C. Profiling of tyrosine phosphorylation pathways in human cells using mass spectrometry. *Proceedings of the National Academy of Sciences of the United States of America*; **2003**, *100*(2), pp 443–448.
47. Brill, L. M.; Salomon, A. R.; Ficarro, S. B.; Mukherji, M.; Stettler-Gill, M.; Peters, E. C. Robust phosphoproteomic profiling of tyrosine phosphorylation sites from human T cells using immobilized metal affinity chromatography and tandem mass spectrometry. *Anal. Chem.* **2004**, *76*(10), 2763–2772.
48. Arnott, D.; Kishiyama, A.; Luis, E. A.; Ludlum, S. G.; Marsters, J. C.; Stults, J. T. Selective detection of membrane proteins without antibodies—A mass spectrometric version of the Western blot. *Mol. Cell. Proteom.* **2002**, *1*(2), 148–156.
49. Henzel, W. J.; Billeci, T. M.; Stults, J. T.; Wong, S. C.; Grimley, C.; Watanabe, C. Identifying Proteins from 2-Dimensional Gels by Molecular Mass Searching of Peptide-Fragments in Protein-Sequence Databases. *Proceedings of the National Academy of Sciences of the United States of America*; **1993**, pp 5011–5015.
50. Shevchenko, A.; Jensen, O. N.; Podtelejnikov, A. V.; Sagliocco, F.; Wilm, M.; Vorm, O.; Mortensen, P.; Boucherie, H.; Mann, M. Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. *Proceedings of the National Academy of Sciences of the United States of America*; **1996**, pp 14440–14445.
51. Gygi, S. P.; Corthals, G. L.; Zhang, Y.; Rochon, Y.; Aebersold, R. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proceedings of the National Academy of Sciences of the United States of America*; **2000**, pp 9390–9395.
52. Simpson, D. C.; Smith, R. D. Combining capillary electrophoresis with mass spectrometry for applications in proteomics. *Electrophoresis* **2005**, *26*(7/8), 1291–1305.
53. Link, A. J.; Eng, J.; Schieltz, D. M.; Carmack, E.; Mize, G. J.; Morris, D. R.; Garvik, B. M.; Yates, J. R. Direct analysis of protein complexes using mass spectrometry. *Nat. Biotechnol.* **1999**, *17*(7), 676–682.
54. Washburn, M. P.; Wolters, D.; Yates, J. R. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* **2001**, *19*(3), 242–247.
55. Peng, J. M.; Elias, J. E.; Thoreen, C. C.; Licklider, L. J.; Gygi, S. P.; Evaluation of multidimensional chromatography coupled with tandem mass spectrometry (LC/LC-MS/MS) for large-scale protein analysis: The yeast proteome. *J. Proteome Res.* **2003**, *2*(1), 43–50.
56. Barnidge, D. R.; Tschumper, R. C.; Jelinek, D. F.; Muddiman, D. C.; Kay, N. E. Protein expression profiling of CLL B cells using replicate off-line strong cation exchange chromatography and LC-MS/MS. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2005**, *819*(1), 33–39.
57. Chiu, D. T.; Wilson, C. F.; Ryttsen, F.; Stromberg, A.; Farre, C.; Karlsson, A.; Nordholm, S.; Gaggari, A.; Modi, B. P.; Moscho, A.; Garza-Lopez, R. A.; Orwar, O.; Zare, R. N. Chemical transformations in individual ultrasmall biomimetic containers. *Science* **1999**, *283*(5409), 1892–1895.
58. Stromberg, A.; Karlsson, A.; Ryttsen, F.; Davidson, M.; Chiu, D. T.; Orwar, O. Microfluidic device for combinatorial fusion of liposomes and cells. *Anal. Chem.* **2001**, *73*(1), 126–130.
59. Garden, R. W.; Moroz, L. L.; Moroz, T. P.; Shippey, S. A.; Sweedler, J. V. Excess salt removal with matrix rinsing: Direct peptide profiling of neurons from marine invertebrates using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *J. Mass Spectrom.* **1996**, *31*(10), 1126–1130.
60. Shimizu, M.; Ojima, N.; Ohnishi, H.; Shingaki, T.; Hirakawa, Y.; Masujima, T. Development of the single-cell MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass-spectroscopic assay. *Anal. Sci.* **2003**, *19*(1), 49–53.
61. Wheeler, A. R.; Thronset, W. R.; Whelan, R. J.; Leach, A. M.; Zare, R. N.; Liao, Y. H.; Farrell, K.; Manger, I. D.; Daridon, A. Microfluidic device for single-cell analysis. *Anal. Chem.* **2003**, *75*(14), 3581–3586.
62. Hu, S.; Zhang, L.; Newitt, R.; Aebersold, R.; Kraly, J. R.; Jones, M.; Dovichi, N. J. Identification of proteins in single-cell capillary electrophoresis fingerprints based on comigration with standard proteins. *Anal. Chem.* **2003**, *75*(14), 3502–3505.
63. Hu, S.; Michels, D. A.; Fazal, M. A.; Ratisoontorn, C.; Cunningham, M. L.; Dovichi, N. J. Capillary sieving electrophoresis/micellar electrokinetic capillary chromatography for two-dimensional protein fingerprinting of single mammalian cells. *Anal. Chem.* **2004**, *76*(14), 4044–4049.
64. Xiao, Y. X.; Feng, Y. P.; Da, S. L.; Yeung, E. S. Progress in single-cell analysis by capillary electrophoresis. *Prog. Chem.* **2004**, *16*(4), 543–553.
65. Hummon, A. B.; Sweedler, J. V.; Corbin, R. W. Discovering new neuropeptides using single-cell mass spectrometry. *Trac-Trends Anal. Chem.* **2003**, *22*(9), 515–521.
66. Li, L. J.; Garden, R. W.; Romanova, E. V.; Sweedler, J. V. In situ sequencing of peptides from biological tissues and single cells using MALDI-PSD/CID analysis. *Anal. Chem.* **1999**, *71*(24), 5451–5458.
67. Li, L. J.; Garden, R. W.; Sweedler, J. V. Single-cell MALDI: A new tool for direct peptide profiling. *Trends Biotechnol.* **2000**, *18*(4), 151–160.
68. Koefler, H. P.; Golde, D. W. Human Myeloid-Leukemia Cell Lines—A Review. *Blood* **1980**, *56*(3), 344–350.
69. Chen, X. Y.; Westphall, M. S.; Smith, L. M. Mass spectrometric analysis of DNA mixtures: Instrumental effects are responsible for decreased sensitivity with increasing mass. *Abstracts Am. Chem. Soc.* **2003**, *226*, U112.
70. Li, L. J.; Romanova, E. V.; Rubakhin, S. S.; Alexeeva, V.; Weiss, K. R.; Vilim, F. S.; Sweedler, J. V. Peptide profiling of cells with multiple gene products: Combining immunochemistry and MALDI mass spectrometry with on-plate microextraction. *Anal. Chem.* **2000**, *72*(16), 3867–3874.
71. Strohl, W. A. Role of Cell Cycle in Abortive Infection of BHK21 Cells with an Oncogenic Adenovirus. *Proceedings of the American Association for Cancer Research*; Boston, MA, April 1970; p 77.
72. Marshall, A. G.; Wang, T. C. L.; Ricca, T. L. Tailored Excitation for Fourier-Transform Ion-Cyclotron Resonance Mass-Spectrometry. *J. Am. Chem. Soc.* **1985**, *107*(26), 7893–7897.
73. Guan, S. H.; Marshall, A. G. Stored waveform inverse Fourier transform (SWIFT) ion excitation in trapped-ion mass spectrometry: Theory and applications. *Int. J. Mass Spectrom. Ion Processes* **1996**, *158*, 5–37.
74. Wang, T. C. L.; Ricca, T. L.; Marshall, A. G. Extension of Dynamic-Range in Fourier-Transform Ion-Cyclotron Resonance Mass-Spectrometry via

- Stored Wave-Form Inverse Fourier-Transform Excitation. *Anal. Chem.* **1986**, 58(14), 2935–2938.
75. Chen, L.; Marshall, A. G. Stored Wave-Form Simultaneous Mass-Selective Ejection Excitation for Fourier-Transform Ion-Cyclotron Resonance Mass-Spectrometry. *Int. J. Mass Spectrom. Ion Processes* **1987**, 79(1), 115–125.
76. Flora, J. W.; Hannis, J. C.; Muddiman, D. C. High-mass accuracy of product ions produced by SORI-CID using a dual electrospray ionization source coupled with FTICR mass spectrometry. *Anal. Chem.* **2001**, 73(6), 1247–1251.
77. Guo, X. H.; Grutzmacher, H. F.; Nibbering, N. M. M. Reactivity and structures of hydrogenated carbon cluster ions $C_nH_x^+$ ($n = 8, 20$; $x = 4–12$) derived from polycyclic aromatic hydrocarbons toward benzene: Ion/molecule reactions as a probe for ion structures. *Eur. J. Mass Spectrom.* **2000**, 6(4), 357–367.
78. Gauthier, J. W.; Trautman, T. R.; Jacobson, D. B. Sustained Off-Resonance Irradiation for Collision-Activated Dissociation Involving Fourier-Transform Mass-Spectrometry Collision-Activated Dissociation Technique that Emulates Infrared Multiphoton Dissociation. *Anal. Chim. Acta* **1991**, 246(1), 211–225.
79. Tsybin, Y. O.; Witt, M.; Baykut, G.; Hakansson, P. Electron capture dissociation Fourier transform ion cyclotron resonance mass spectrometry in the electron energy range 0–50 eV. *Rapid Commun. Mass Spectrom.* **2004**, 18(14), 1607–1613.
80. Gorshkov, M. V.; Masselon, C. D.; Nikolaev, E. N.; Udseth, H. R.; Pasa-Tolic, L.; Smith, R. D. Considerations for electron capture dissociation efficiency in FTICR mass spectrometry. *Int. J. Mass Spectrom.* **2004**, 234(1/3), 131–136.
81. Stensballe, A.; Jensen, O. N.; Olsen, J. V.; Haselmann, K. F.; Zubarev, R. A. Electron capture dissociation of singly and multiply phosphorylated peptides. *Rapid Commun. Mass Spectrom.* **2000**, 14(19), 1793–1800.
82. Hakansson, K.; Emmett, M. R.; Hendrickson, C. L.; Marshall, A. G. High-sensitivity electron capture dissociation tandem FTICR mass spectrometry of microelectrosprayed peptides. *Anal. Chem.* **2001**, 73(15), 3605–3610.
83. Syka, J. E. P.; Coon, J. J.; Schroeder, M. J.; Shabanowitz, J.; Hunt, D. F. Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. *Proceedings of the National Academy of Sciences of the United States of America*; 2004; pp 9528–9533.
84. Coon, J. J.; Shabanowitz, J.; Hunt, D. F.; Syka, J. E. P. Electron transfer dissociation of peptide anions. *J. Am. Soc. Mass Spectrom.* **2005**, 16(6), 880–882.
85. Lin, M.; Campbell, J. M.; Mueller, D. R.; Wirth, U. Intact protein analysis by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **2003**, 17(16), 1809–1814.
86. Sadygov, R. G.; Cociorva, D.; Yates, J. R. Large-scale database searching using tandem mass spectra: Looking up the answer in the back of the book. *Nat. Methods* **2004**, 1(3), 195–202.