Exploiting the Complementary Nature of LC/MALDI/MS/MS and LC/ESI/MS/MS for Increased Proteome Coverage

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The goal of this work was to evaluate the improvement in proteome coverage of complex protein mixtures gained by analyzing samples using both LC/ESI/MS/MS and LC/MALDI/MS/MS. Parallel analyses of a single sample were accomplished by interfacing a Probot fractionation system with a nanoscale LC system. The Probot was configured to perform a post-column split such that a fraction (20%) of the column effluent was sent for on-line LC/ESI/MS/MS data acquisition, and the majority of the sample (80%) was mixed with a matrix solution and deposited onto the MALDI target plate. The split-flow approach takes advantage of the concentration sensitive nature of ESI and provides sufficient quantity of sample for MALDI/MS/MS. Hybrid quadrupole time-of-flight mass spectrometers were used to acquire LC/ESI/MS/MS data and LC/MALDI/MS/MS data from a tryptic digest of a preparation of mammalian mitochondrial ribosomes. The mass spectrometers were configured to operate in a data dependent acquisition mode in which precursor ions observed in MS survey scans are automatically selected for interrogation by MS/MS. This type of acquisition scheme maximizes the number of peptide fragmentation spectra obtained and is commonly referred to as shotgun analysis. While a significant degree of overlap (63%) was observed between the proteins identified in the LC/ESI/MS/MS and LC/MALDI/MS/MS data sets, both unique peptides and unique proteins were observed by each method. These results demonstrate that improved proteome coverage can be obtained using a combination of these ionization techniques. (J Am Soc Mass Spectrom 2003, 14, 971–979) © 2003 American Society for Mass Spectrometry

The most significant figure of merit in proteome analysis is the extent of proteome coverage. Proteomic samples can be exceedingly complex—a proteolytic digest of a simple cell lysate can contain several hundred thousand peptides. Nanoscale capillary LC and electrospray ionization (ESI) coupled with data dependent MS/MS acquisition has been shown [1–7] to be an exceptionally useful tool for the analysis of complex proteomic samples, yielding high sensitivity (low femtomoles to high attomoles), high data acquisition rate (1000 precursors/h), and high information content (superior quality MS/MS spectra). Matrix assisted laser desorption ionization (MALDI) in conjunction with MS/MS analysis has also been used to acquire product ion spectra from proteomic samples, generating data sets both analogous to and complementary with those produced by LC/ESI/MS/MS [8–12]. While differences in the spectral content of MALDI/MS/MS and ESI/MS/MS of the same peptides have been described [9, 10, 13], there has been less discussion focused on findings that analysis of the same sample by both LC/ESI/MS/MS and LC/MALDI/MS/MS gives rise to improved proteome coverage at both the peptide and protein level.

Despite advances in software and instrumentation that have enabled rapid acquisition of very large data sets, the extreme complexity of proteomic samples typically leads to peptides being introduced into the mass spectrometer at a rate far exceeding that of MS/MS data acquisition [14, 15]. This results in an under sampling of the proteome at least at the peptide level, and most often also at the protein level. Increasing the number of unique peptide fragmentation spectra acquired for database searching should lead to the identification of a larger number of proteins (proteome coverage) as well as greater confidence in the proteins identified (sequence coverage). Furthermore, the con-

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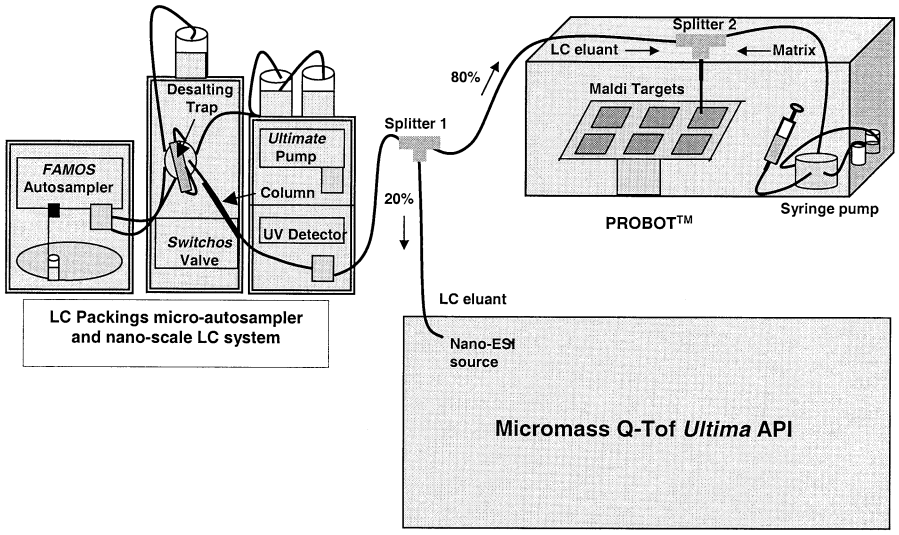

Figure 1. Probot fractionation system coupled to a Q-TOF *Ultima* API instrument (http://www.micromass.co.uk) for on-line LC/ESI/MS/MS and parallel LC/MALDI/MS/MS.

stant expansion of protein and gene databases demands an increase in the number of peptide spectra required for unambiguous assignment of proteins.

One means by which increased proteome coverage can be achieved is through the use of multiple dimensions of chromatography (LC/LC). In a typical LC/LC/MS/MS experiment, a complex protein or peptide mixture is separated in the first dimension by strong cation exchange chromatography followed by reversed phase separation in the second dimension. Reports from our lab and others [16–20] have demonstrated the use of LC/LC/ESI/MS/MS to acquire data from several thousand proteins per sample. An alternate approach described here takes advantage of the complementary nature of ESI and MALDI and utilizes a split-flow, parallel analysis scheme with data dependent MS/MS acquisitions. This combined technique facilitates the interrogation of both multiply (produced by ESI) and singly (produced by MALDI) charged precursor ions and provides complementary information for database searching [8, 9]. Medzihradszky et al. [21] and Juhasz et al. [22] have reported similar findings in which more comprehensive characterization of complex protein digests were obtained by using ESI/MS/MS, MALDI/MS, and/or MALDI/MS/MS analyses.

Here we report the identification of proteins from the large, 39S subunit of bovine mitochondrial ribosomes via the combination of LC/ESI/MS/MS and LC/MALDI/MS/MS analyses. Proteins synthesized in mammalian mitochondria are incorporated into complexes that are responsible for oxidative phosphorylation and the synthesis of the majority of the ATP in these organisms. The human 39S subunit has been reported to contain at least 48 distinct proteins, with over half of these being homologous to ribosomal proteins from other species [15]. The application of complementary ionization techniques in the analysis of a tryptic digest of the bovine 39S subunit has led to the identification of 51 ribosomal proteins. Of the 51 proteins identified, 32 were found by both techniques, while 8 were unique to the LC/ESI/MS/MS analysis and 11 to the LC/MALDI/MS/MS analysis.

Table 1. Ribosomal proteins identified from bovine mitochondria using coupled LC/ESI and LC/MALDI MS/MS analyses and subsequent MS/MS ion searches of a non-redundant protein database

Protein name	LC/ESI/ MS/MS	LC/MALDI/ MS/MS	Protein name	LC/ESI/ MS/MS	LC/MALDI/ MS/MS
L1	X		S2		X
L2	X	X	S3A	X	
L3	X		S5	X	
L4	X	X	S6	X	
L7		X	S7	X	X
L9	X		S9	X	
L11	X	X	S11	X	
L12	X	X	S12		X
S13	X	X	S14	X	
L15		X	S15	X	X
L16	X	X	S18-2	X	X
L17		X	S18-3		X
L18-1	X		S20	X	
L19		X	S21	X	
L20	X		S22	X	X
L22		X	S23		X
L26		X	S24	X	
L32	X	X	S25	X	
L33	X		S27	X	X
L36-A		X	S28	X	
L39	X	X	S29	X	X
L45	X	X	S30	X	X
L47	X	X	S31	X	X
L49	X	X	S34	X	X
L50	X	X	S35	X	
L56	X	X			

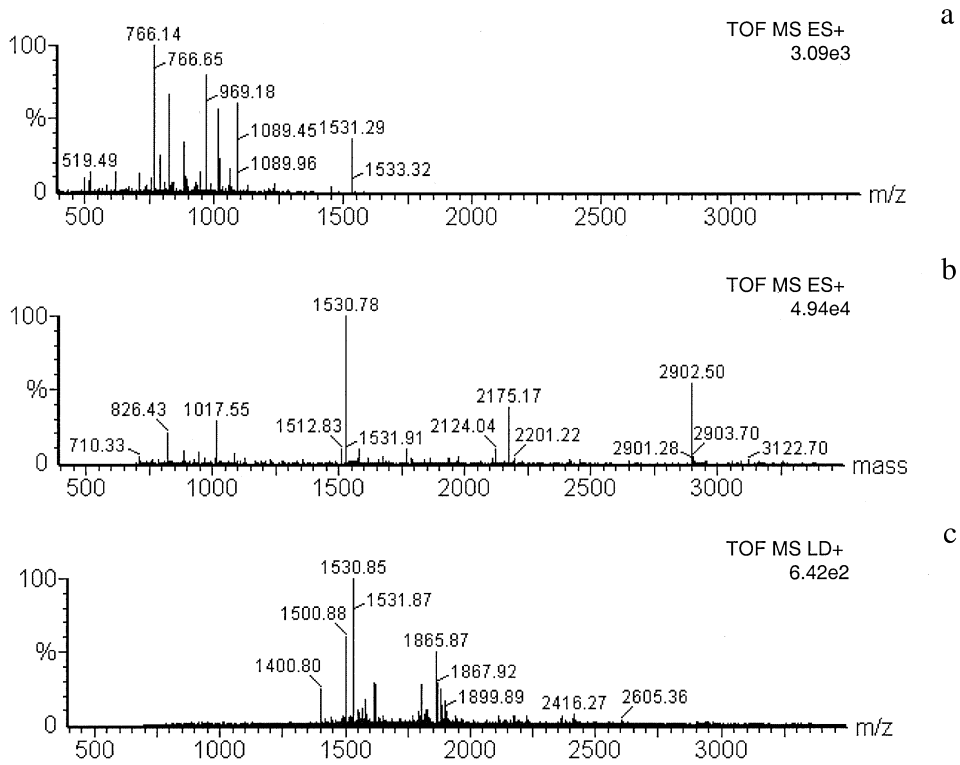

Figure 2. Comparison of the LC/ESI/MS spectrum (**a**) and MaxEnt transform to singly charged ions (**b**) with the LC/MALDI/MS spectrum (**c**) for a 1-min fraction.

Experimental

Chemicals and Reagents

Analytical grade reagents and chemicals were obtained from Sigma (St. Louis, MO) and HPLC grade solvents from Burdick and Jackson (Muskegon, MI). Sequencing grade trypsin (porcine) was obtained from Promega (Madison, WI).

Sample Preparation

Bovine mitochondrial ribosomes were prepared according to the procedure described by Spremulli and coworkers [23]. Peptides were generated from this complex mixture of ribosomal proteins by digestion in solution with a 1:10 ratio of protein:trypsin by weight. Prior to digestion, protein disulfide bonds had been reduced and alkylated with dithiothreitol and iodoacetamide (Sigma).

Liquid Chromatography

Nanoscale capillary LC separations were performed with an Ultimate nanoscale LC system, a FAMOS micro-autosampler and Switchos valves from LC Packings (Amsterdam, The Netherlands). Sample pre-concentration and desalting was accomplished with a 1mm pre-column cartridge (LC Packings), and separations were performed with a 180 um i.d. × 15 cm l. PepMap C18 column (LC Packings). The gradient pump was programmed to deliver 5 to 40% acetonitrile over 180 min at a flow rate of 1.5 uL/min. Mobile phase A consisted of 0.1% formic acid in 2:98 acetonitrile:water (vol/vol) and mobile phase B consisted of 0.1% formic acid in 95:5 acetonitrile:water (vol/vol). The system was configured to perform a post-column split such that 200 nL/min was introduced to the nano-electrospray source of the mass spectrometer while 1.3 uL/min was delivered to an on-line Probot (Dionex, Sunnyvale, CA) system. A schematic of the Probot fractionation system is shown in Figure 1. Eluant was coaxially mixed with a solution of MALDI matrix (7mg/mL αcyano-4-hydroxycinnamic acid) and deposited as discrete spots on MALDI targets. Each spot represented a 1-min "fraction" of a 3-h reversed phase gradient.

Mass Spectrometry

Hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometers were used to acquire both LC/ESI/MS/MS spectra (Q-TOF Ultima API, Micromass UK Ltd.) and LC/MALDI/MS and LC/MALDI/MS/MS spectra (Q-TOF Ultima MALDI, Micromass UK Ltd.). Data dependent LC/ESI/MS/MS acquisitions were performed as described previously [24], with up to eight precursors selected for interrogation from each MS survey scan. Precursor selection was based upon ion intensity, charge state, and if the precursor had been previously selected for interrogation (dynamic exclusion). One collision energy was used for each precursor,

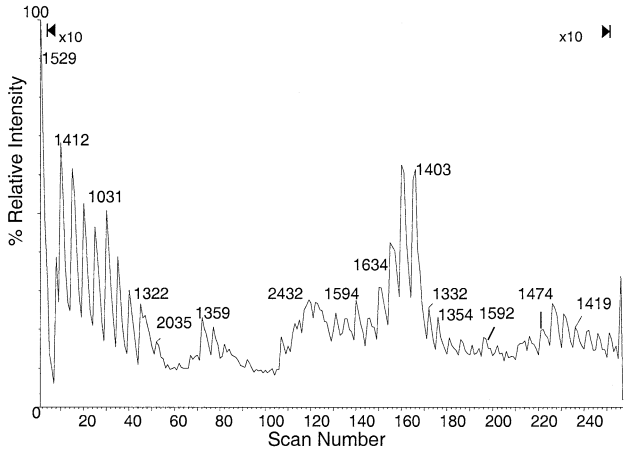

Figure 3. Representative ion trace from the MALDI/MS/MS of a single one-min LC fraction, labeled with the *m/z* values for the 15 precursor ions subjected to MS/MS. The "sawtooth" pattern results from the use of as many as five different collision energies for each precursor.

with the collision energy selected on the basis of ion charge state and *m/z* value. For data dependent LC/MALDI/MS/MS acquisitions, the mass spectrometer was set to acquire positive ion MS survey scans over the mass range of 700–3500 Da. Each MS spectrum was automatically combined with previous scans into a rolling-average, and this process continued until a peak within a user-defined "Peak Monitor Window" (1000–2500 Da) reached an intensity value greater than a user-defined threshold. Once the MS survey scans were completed, the data were processed automatically to generate a list of ions for interrogation by MS/MS. The operator has the flexibility to define a "Peak Switch Window" such that any peaks falling outside this window (for example matrix ion clusters) would not be subjected to MS/MS. The list of candidate ions for MS/MS was ordered based on decreasing intensity and compared against the include, exclude, and adduct lists prior to generation of the final "Set Mass List" for automated MS/MS. The MS/MS spectra were acquired in a similar fashion in which spectra were combined, and the rolling-average was inspected for a fragment

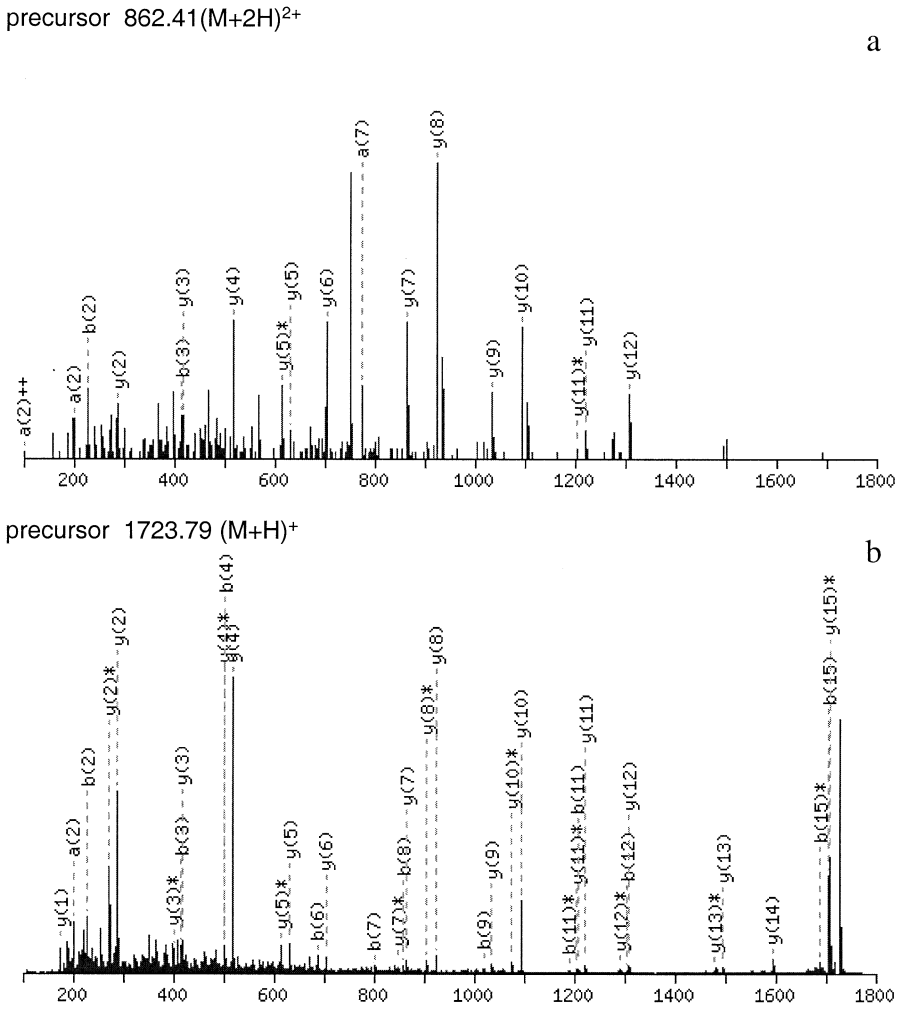

Figure 4. Comparison of LC/ESI/MS/MS (**a**) and LC/MALDI/MS/MS (**b**) spectra for peptide EVWSEGLGYADVENR from 39S ribosomal protein L56.

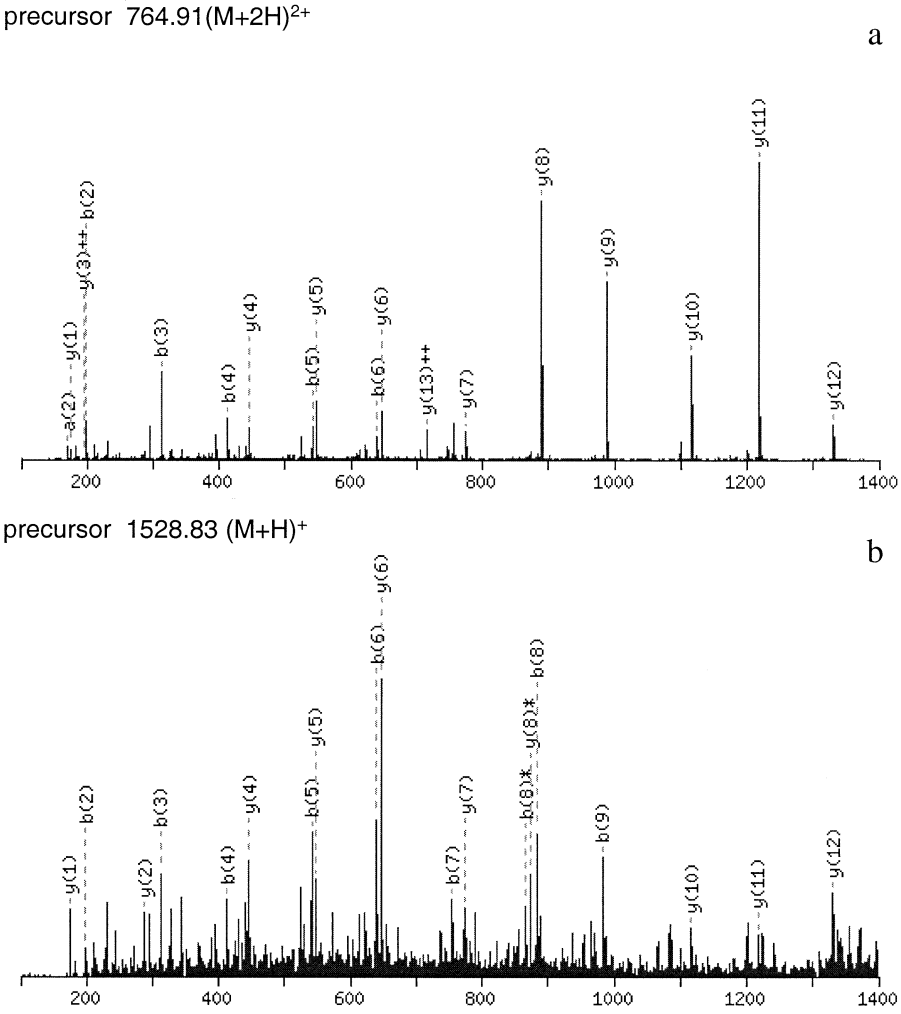

Figure 5. Comparison of LC/ESI/MS/MS (**a**) and LC/MALDI/MS/MS (**b**) spectra for peptide TPITQVNEVTGTLR from 60S ribosomal protein L49.

ion intensity above a user-defined threshold. The operator has the flexibility to define a "Monitor Window" such that the precursor ion intensity will not be considered. A collision energy profile (five collision energy values per precursor) was used to ensure that the optimal degree of fragmentation was achieved. For a given sample, the MS/MS acquisitions stopped when one of the following conditions was met: Either the end of the precursor list was reached; the sample had been depleted (end of laser firing pattern); or the total time set for acquisition had expired. Single point "Lock Mass" correction was employed using Glu-fibrinopeptide (*m/z* 1570.6774) as the near point (external) calibration standard to reduce mass errors down to 5–10 parts per million (ppm).

Data Processing and Database Searching

MassLynx version 3.5 (http://www.micromass.co.uk) was used to process raw MS/MS data prior to database searching. Data were "quality-filtered" such that spectra containing insufficient information for peptide mapping were removed prior to database searching. Peak list files were searched against a non-redundant protein database using the Mascot search engine (http://www.matrixscience.com) versions 1.7 (in-house on a 20 processor cluster) and 1.8 (web-based).

Results and Discussion

Analysis of bovine mitochondrial ribosomes was accomplished using a split-flow approach with on-line LC/ESI/MS/MS analysis and parallel LC/MALDI/MS/MS. A total digest of the 39S subunit was separated by a 3 h reversed phase gradient with 20% of the column effluent sent directly to the ESI source and 80% of the sample mixed with MALDI matrix and spotted in 1 min fractions on a MALDI target. Both mass spectrometer systems were configured to operate in data dependent acquisition or "shotgun analysis" mode to maximize the number of precursors interrogated. The ribosomal proteins identified are listed in Table 1. Examination of the results revealed the presence of proteins from both the large (39S) and small (28S)

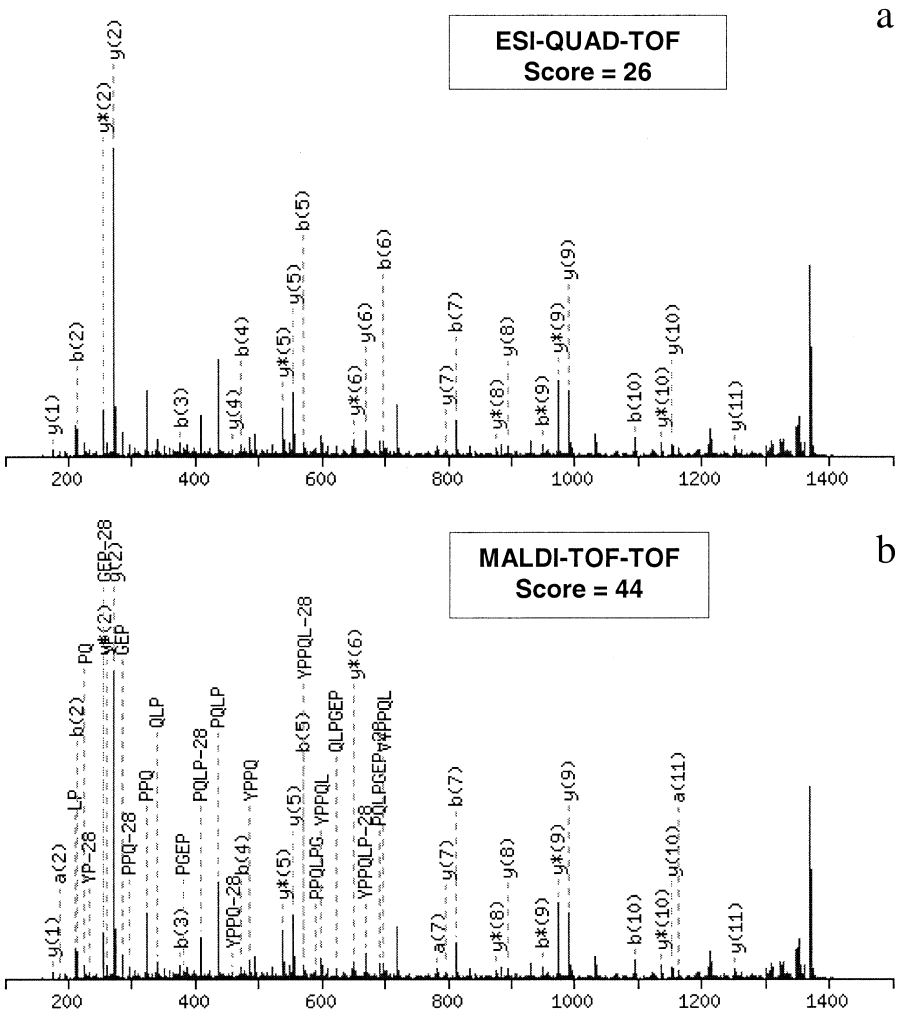

Figure 6. Comparison of fragment ions detected from Mascot version 1.8 when the LC/MALDI/MS/MS spectrum for peptide IVYPPQLPGEPR (ribosomal protein L22) was searched with the instrument parameter set to ESI-QUAD-TOF (**a**) or MALDI-TOF-TOF (**b**).

ribosomal subunits, indicating co-purification of the smaller subunit during isolation of the 39S subunit. Due to the limited number of bovine ribosomal proteins represented in the database, several of the protein assignments were made based on homology to human or mouse ribosomal proteins. A significant number (>10) of co-purified contaminant proteins (non-ribosomal) were also observed.

A comparison of the LC/ESI/MS and LC/MALDI/MS survey scans representing a 1-min fraction of the gradient is shown in Figure 2. There are a number of peptides observed by both techniques, however, the peptide ion signals are generally more intense in the ESI/MS spectra (base peak intensity approximately 5-fold greater). The difference in absolute abundance as well as signal to noise can be attributed in part to differences in the ionization processes and in part to the experimental method. In the MALDI data dependent acquisition, the method is set to acquire a minimum number of MS scans in order to conserve the majority of the sample for interrogation by MS/MS. For example, in the ion chromatogram shown in Figure 3, there were approximately 22 min (260 scans) spent acquiring MS/MS data on 15 precursors that were selected based on only 10 s (2 scans) of MS survey data. For comparison, there were 7 multiply charged precursors subjected to MS/MS in the ESI experiment from the corresponding 1-min period (data not shown). The formation of matrix ion clusters and adducts during ionization can contribute to the background signal and in some cases lead to ion suppression in the MALDI/MS experiment. More rigorous inspection of the LC/ESI/MS spectrum for a longer interval (3 min) and the corresponding 3 MALDI/MS spectra (each MALDI spot equals 1 min) revealed that 3 peptides were observed in the ESI/MS spectrum but the not in the corresponding MALDI/MS spectra, 6 peptides were observed by MALDI, but not by ESI, and 16 peptides were observed by both methods (data not shown).

Representative fragment ion spectra for two peptides that were observed in both the LC/ESI/MS/MS and LC/MALDI/MS/MS analyses are shown in Figures 4

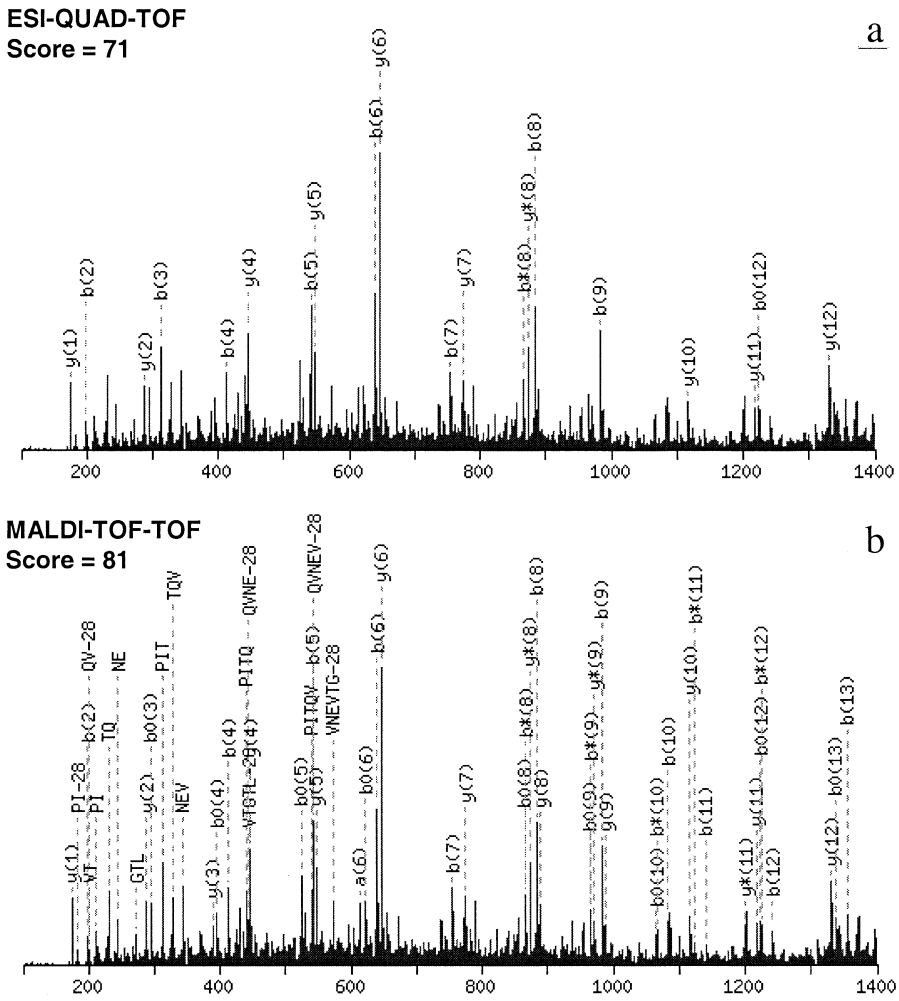

Figure 7. Comparison of fragment ions detected from Mascot v1.8 when the LC/MALDI/MS/MS spectrum for peptide TPITQVNEVTGTLR (ribosomal protein L49) was searched with the instrument parameter set to ESI-QUAD-TOF (**a**) or MALDI-TOF-TOF (**b**).

and 5. In each case, the peptide mass error obtained was less than or equal to 0.03 Da, and the Mascot scores were in the confidence range of 95% probability or better. As expected, there were differences observed in the fragmentation patterns and fragment ion intensities between the doubly charged peptide ions produced by ESI and the singly charged ions produced by MALDI. In general, the corresponding multiply charged peptides tended to produce cleaner spectra with less internal fragments, and in some cases, less structural information. In MS/MS experiments, the degree of fragmentation varies with amino acid sequence, but it can be modulated by varying the collision energy. In the LC/MALDI/MS/MS method, as many as five different voltage settings were used in the collision energy profile in order to achieve optimal fragmentation of the singly charged precursors. In Figure 4b however there is a considerable amount of precursor ion remaining. It has been our observation with the Q-TOF Ultima MALDI system that increasing the collision energy for some peptide ions leads to a reduction in precursor ion signal without the concomitant increase in fragment ion signal.

Processed data files were initially searched against a non-redundant protein database using the Matrix Science search engine, Mascot version 1.7. Following the release of Mascot version 1.8 (http://www.matrixscience.com), searches were repeated with inclusion of a parameter setting for the instrument type (e.g., ESI-QUAD-TOF, MALDI-TOF-TOF, etc.). This parameter defines the degree of fragmentation expected for a set of peptides based on either the low or high-energy collisions that result from a particular instrument design. Interestingly, comparisons of MS/MS ion search results using Mascot version 1.7 and version 1.8 (including the instrument parameter) show significant improvements (up to 70%) in score with Mascot version 1.8 when MALDI Q-TOF data is searched as MALDI/TOF/TOF data. This finding suggests that a higher degree of fragmentation is obtained for the singly charged peptide ions in a MALDI Q-TOF instrument than was expected. As shown in Figures 6 and 7, the fragmenta-

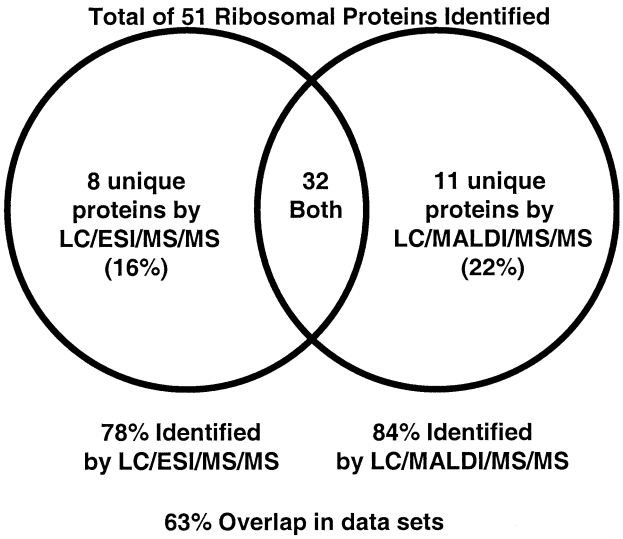

Figure 8. Comparison of the number of overlapping and unique protein assignments obtained by LC/ESI/MS/MS and LC/MALDI/MS/MS analysis of the 39S subunit of bovine mitochondrial ribosomes.

tion of peptides in the MALDI Q-TOF yields significant amounts of internal fragments and neutral losses (including a, y*, and b* type ions). In the example shown, a substantial proportion of ion current could be attributed to these higher energy fragments resulting in a 69% improvement in Mascot score. Elevated Mascot scores indicate higher probability and thus greater confidence that the proteins identified are not merely random matches in the database. It has been noted that analysis of peptides using MALDI/TOF/TOF instruments using high energy collisions (>1 kV) gives rise to a significant number of similar fragments [10, 21]. The d and w ions generated in high energy collisions were not observed however in the MALDI Q-TOF data.

While there was a significant overlap observed between the two data sets, a considerable number of unique proteins were observed by either LC/ESI/MS/MS or LC/MALDI/MS/MS (Figure 8). This finding indicates that improved proteome coverage was obtained using a combination of the two techniques, and is in agreement with other reports (Patterson, D. H.; Vestal, M.; Martin, S., personal communication, 2002). The number of peptide matches corresponding to a given protein assignment varied from as few as 1 to as many as 13. During the LC/MALDI/MS/MS acquisitions, some percentage of time was spent interrogating peptides that had been analyzed in previous sample spots. Dynamic exclusion of precursor ions that have been interrogated in the preceding sample spot would be a useful addition to the current acquisition software as it should lead to the acquisition of a greater number of unique MS/MS spectra and potentially a further increase in proteome coverage.

Conclusions

We have demonstrated the use of a Probot fractionation system with a post-column splitter to couple LC separation with on-line ESI/MS/MS and parallel MALDI/MS/MS for proteomic analysis of complex mixtures. Examination of a proteolytic digest of the large, 39S subunit of bovine mitochondrial ribosomes led to the identification of a total of 51 proteins with a 63% overlap in assignments made by the two different techniques. While application of the method described in this publication did generate complementary data sets and unique protein identifications, more complete proteome coverage will likely require a more intelligent use of both LC/ESI/MS/MS and LC/MALDI/MS/MS. An ideal integration of the two techniques would incorporate the post-column split with on-line LC/ESI/MS/MS analysis and subsequent, targeted MALDI/MS/MS analysis of peptides not sampled by LC/ESI/MS/MS. This approach would not require division of the sample prior to analysis and would take advantage of the concentration sensitive nature of ESI while maximizing the amount of sample available for targeted MALDI/MS/MS. Further advances in the sensitivity (product ion intensity per unit time) of MALDI/MS/MS to make it comparable to that of ESI/MS/MS, should facilitate interrogation of an even greater number of peptides and ultimately lead to increased proteome coverage and resolution of ambiguities in the identification of splice variants and other homologous proteins.

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