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Monolithic Column HPLC Separation of Intact Proteins Analyzed by LC-MALDI Using On-Plate Digestion: An Approach To Integrate Protein Separation and Identification

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A method is developed to integrate a protein separation by monolithic capillary reversed-phase high-performance liquid chromatography to on-probe tryptic digestion for subsequent analyses by MALDI-TOF MS and MALDI-TOF/TOF MS. The method provides a means of directly interfacing separations to MALDI-MS, reducing the amount of time required for traditional procedures involving in-solution enzymatic digestion and sample cleanup prior to MALDI-MS analysis. When used with pI-based fractionation as a first dimension, it provides a means of analyzing complex mixtures of proteins with minimal sample handling and cleanup. The use of monolithic capillary columns sufficiently resolved intact proteins so that peptide mass fingerprinting analysis by MALDI-TOF MS resulted in the identification of close to 40 unique proteins from 120 ng of sample obtained from a prefractionated MCF10 cell line at pH 6.34, where the identifications of several of these proteins were also confirmed by intact MW and tandem mass spectrometric analysis. The reproducibility of this method has been demonstrated to be sufficient for the purpose of protein identifications. Experimental values of protein intact MW are obtained and compared to that expected for each protein identified.

The development of multidimensional high-performance liquid chromatography (HPLC) separation in proteomics has greatly contributed to simplifying sample purification procedures for the analysis of highly complex biological mixtures. The effluent from the reversed-phase (RP)-HPLC can be analyzed by on-line ESI-MS or, alternatively, can be collected for off-line MALDI-based MS analysis. In either case, such mass spectrometric analysis is generally performed using in-solution tryptic digestion and sample purification of the peptide map that results. These extensive off-line sample procedures are a drawback, where enzymatic digestion typically occurs overnight and sample purification with commercially available SPE-based C18 Ziptips is tedious and prone to the loss of peptides of extreme hydrophilicity.[1] In addition, loss of peptides due to repetitive sample transfers is inevitable and leads to low sequence coverage for database searching for less confident protein identification. This series of time-consuming and laborious techniques is not suitable for large-scale proteomic study of samples of high complexity where hundreds of unique proteins are to be analyzed. Thus, the development of rapid and high-throughput techniques to accelerate enzymatic digestion and sample purification for reliable peptide mass fingerprinting (PMF) analysis is needed.

Various methods have been proposed to expedite the enzymatic digestion processes, including microwave-assisted digestion[2−4] and on-line tryptic digestion.[5−15] Microwave-assisted digestion can accelerate enzymatic digestion into minutes using a very high concentration of acid to assist the hydrolysis of peptides. An on-line trypsin digestion combined with protein separation was

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demonstrated,[8] although the application has been limited mainly to standard proteins. Enzymatic digestion on-plate MALDI[16,17] is a promising method to significantly reduce the time required for tryptic digestion to obtain high-throughput analysis. The on-plate digestions reported so far, however, were mostly performed on standard protein mixtures or cells from very simple organisms.

Microscale HPLC provides rapid separation of proteins; in particular, the monolithic capillary column has drawn much attention in proteomic studies due to its high separation speed, high efficiency, and high recovery.[18,19] Moreover, its low flow rate, typically in the range of a few microliters per minute, is ideally suited to be combined with off-line peak collections for MALDI MS for high-throughput analysis. A heated droplet interface[20] and monolithic capillary LC-MALDI[21] have been reported to couple microscale HPLC for peptide separations for subsequent tandem MALDI MS analysis.

In this work, two-dimensional liquid-phase separation was coupled with on-plate digestion of proteins for subsequent MALDI MS to identify the proteins in cell lysates from a human breast cancer cell line MCF10A by PMF analysis. It was demonstrated that off-line analysis and on-plate digestion can be achieved for rapid analysis with minimal sample handling. It was shown that generally high sequence coverage is obtained for close to 40 unique proteins separated by monolith-based HPLC. In addition, the MW values of the proteins identified in this experiment were compared to intact MW values measured using nonporous RP-HPLC separation of the proteins interfaced to ESI-TOF MS as a means to further constrain the MALDI-TOF MS database search.

EXPERIMENTAL SECTION

The experimental overview is described in Figure 1. Proteins from MCF10A were first separated using the Rotofor device for pI-based fractionation, where one of the fractions was selected for further separation by monolithic capillary HPLC. The fractions at 15- and 30-s intervals were collected off-line on the MALDI plate precoated with trypsin for on-plate digestion and subsequent analysis. The same pH fraction is also analyzed by on-line ESI-TOF MS with nonporous (NPS) C18 packed column to obtain intact protein MW values for comparison to theoretical MW values.

Materials and Reagents. Ammonium bicarbonate, trifluoroacetic acid (TFA), formic acid, α-cyano-4-hydroxycinnamic acid (α-CHCA), and acetonitrile were purchased from Sigma (St. Louis, MO). A TPCK-modified trypsin (porcine) of sequencing grade was purchased from Promega (Madison, WI). A protein assay kit and bovine serum albumin standard were obtained from Bio-Rad Laboratories (Hercules, CA). DI water was purified by the Milli-Q water filtration system (Millipore, Inc., Bedford, MA). All the reagents were used without further purification.

Sample Preparation and pI-Based Separation. The cells used in this work include MCF10A, which is maintained and prepared by the Barbara Ann Karmanos Cancer Institute (Wayne State University, Detroit, MI), as previously described.[22] The preparation of cell extracts for liquid-phase IEF and its fractionation using the Mini-Rotofor (Bio-Rad) as well as the protein quantitation based on the Bradford method and pH measurements were performed as described elsewhere.[23]

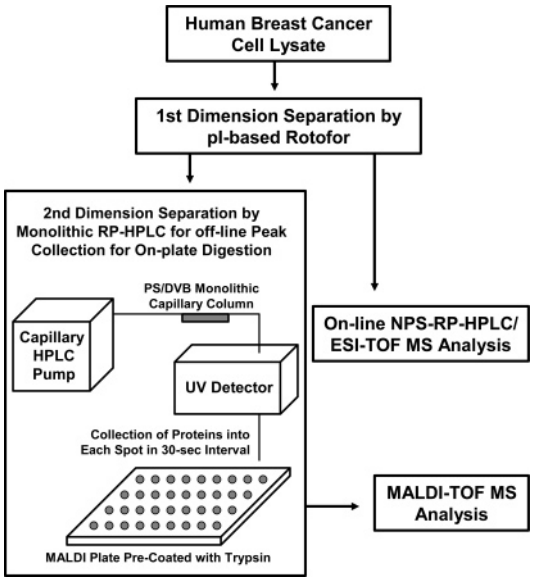

Figure 1. Experimental scheme of the two-dimensional liquid-phase separation followed by on-plate digestion for MALDI-TOF MS analysis for identification of proteins in human breast cancer cell line.

On-Line NPS-RP-HPLC/ESI-TOF MS for Intact MW Analysis. Intact MW analysis was performed as described previously.[23] Briefly, the NPS-RP-HPLC column (33 mm L × 4.6 mm i.d.) packed with 1.5-μm C18 nonporous ODSIIIE silica beads (Eprogen, Darien, IL) was used for the separation of the MCF10 cell line prefractionated at pH 6.34 at a flow rate of 0.5 mL/min. The HPLC System Gold with UV detector set at 214 nm (Beckman Coulter, Fullerton, CA) was used with the solvents A and B composed of 0.3% formic acid in DI water and acetonitrile, respectively. The column was maintained at 65 °C (model 7971 column heater, Jones Chromatography, Resolution Systems, Holland, MI), and the following gradient was used: 5−15% B in 1 min, 15−25% B in 2 min, 25−31% B in 3 min, 31−41% B in 10 min, 41−47% B in 3 min, 47−67% B in 4 min, 67−100% B in 1 min, 100% B for 2 min, and 100−5% B in 1 min. A splitter system was used so that 40% of eluent from HPLC was delivered on-line to ESI-TOF MS (LCT, Waters-Micromass, Manchester, U.K.). The desolvation temperature was set at 300 °C and the source temperature at 120 °C, where nitrogen gas flow was controlled at 650 L/h. One mass spectrum was acquired per second, and the deconvolution of the combined spectra of the protein was performed by utilizing the MaxEnt1 of MassLynx software version 4.0 (Waters-Micromass).

Monolithic Capillary HPLC Separation and On-MALDI Plate Enzymatic Digestion. The preparation of monolithic capillary columns (360 μm o.d. × 200 μm i.d. × 60 mm L) by copolymerizing styrene and divinylbenzene (PS/DVB) was per-

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formed according to procedures described elsewhere.[24] The UltraPlus II MD Capillary Pump module (Micro-Tech Scientific, Vista, CA) was used for the chromatographic separation utilizing a monolithic column. The capillary column was directly mounted to a microinjector with a 500-nL internal sample loop (Valco Instruments, Houston, TX) by a microtight union (Upchurch Scientific, Oak Harbor, WA). The capillary protein separation was controlled at 60 °C with a column heater utilizing a variable autotransformer (Staco Energy Product, Dayton, OH). The flow from the solvent delivery pump was split precolumn in order to produce a flow rate of ~2.5 μL/min through a monolithic capillary column. A mobile-phase system of two solvents was used, wherein solvents A and B were composed of 0.05% formic acid in DI water and acetonitrile, respectively. A linear gradient of 0−100% B in 18 min was applied.

Approximately 120 ng of prefractionated MCF10A cell line at pH 6.34 was loaded onto a monolithic capillary column for separation, which was monitored at 214 nm to acquire chromatograms using a UV detector (Dionex/LC Packings, Sunnyvale, CA) equipped with a 3-nL flow cell. The proteins eluting off the monolithic capillary column were directly deposited onto 96-well and 384-well MALDI plates at 15- and 30-s intervals for each spot. The MALDI plate was precoated with 0.5 μL of trypsin stock solution of ~0.15 μg/μL. Following the sample collection to ensure complete dryness of each spot, 0.5 μL of 50 mM ammonium bicarbonate was added to the top layer of each spot and the plate was kept in a humidifier chamber for 5 min at room temperature for digestion. Then, 0.5 μL of 0.1% TFA was added to each spot to stop the digestion, followed by adding 0.5 μL of α-CHCA matrix solution prepared by diluting saturated α-CHCA with 60% acetonitrile/0.1% TFA at a 1:4 ratio. For the MALDI-TOF MS experiment, the internal standards, including angiotensin I, adrenocorticotropic hormone (ACTH) fragment 1−17, and ACTH fragment 18−39, were added so that a final concentration of 50 fmol of each standard was placed in every spot of the MALDI plate. For the MALDI-TOF/TOF MS experiment, several trypsin autolysis products were used for internal calibrations, while no additional internal standards were included.

MALDI MS Analysis and Database Searching. The MALDI-TOF MS analysis was performed on a TofSpec2E (Waters-Micromass) equipped with delayed extraction in reflectron mode with positive polarity. A 337-nm Nd:YAG laser was used as the ionization source, where the coarse laser energy was set at 50% and the fine laser energy varied from 20 to 90% at the laser frequency of 5 Hz. The delay time was set at 520 ns, the source voltage at 20 kV, the extraction voltage at 1:1 to the source voltage, the pulse voltage at 2300 V, and the reflectron voltage at 24.5 kV, wherein 15−20 spectra were collected over the m/z range of up to 4000 Da. Each spectrum was internally calibrated, and monoisotopic peptide masses were obtained using MassLynx software version 4.0 (Waters-Micromass) for submission to the MS-Fit search engine using SwissProt database (2005.01.06) for protein identification. The search was carried out under the species of *Homo sapiens* at 50 ppm mass tolerance with no limitation set for the molecular weight and pI. The possible modifications included N-terminal Gln to pyroGlu, oxidation of Met, N-terminal acetylation, and phosphorylation at S, T, and Y.

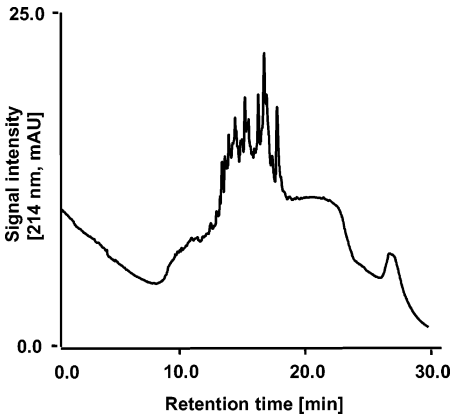

Figure 2. Representative protein separation chromatogram obtained for monolithic capillary LC for detection at 214 nm.

The MALDI-TOF/TOF MS analysis was performed using a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA), where reflector laser shots were set at 750. A S/N threshold of 50 was used for selecting peaks, while 30 was used for MS/MS peak selection. A fragmentation voltage of 2 kV was used throughout the automated runs. An on-line version of MASCOT (www.matrixscience.com) was used for database searching under SwissProt. The search was performed under *Homo sapiens* using a MS tolerance of 50 ppm for precursor ions and 0.3 Da for fragment ions, while allowing one missed cleavage and the same variable modifications as used in PMF analysis.

RESULTS AND DISCUSSION

Protein Separation by Monolithic Capillary Column. Proteins prefractionated based on pI were separated with a PS/DVB monolithic capillary column using a low flow rate of 2.5 μL/min, which is suitable for directly depositing proteins on the MALDI plate. Only ~120 ng of total proteins was consumed for the entire experiment. The actual separation was completed in ~18 min to separate one pH fraction. Compared to the typical 40-min separation time used in packed column capillary HPLC separation, the separation time was reduced significantly by the use of monolith-based microscale HPLC separation. Figure 2 shows a representative protein separation profile obtained for ~120 ng of pI-based Rotofor fraction from human breast cancer cells using a monolithic capillary column, where the overall separation time was substantially less than 30 min. However, the separation efficiencies of the intact proteins were observed to be significantly less than those typically achievable for peptide separations.[25] Although the 200-μm-i.d. monolithic columns may not be sufficient for high-resolution separation of highly complex protein mixtures in human cancer cells, this problem becomes less significant with the detection of protein digests by MALDI-TOF MS, on-plate digestion, and the use of intact MW analysis.

Identification of Proteins by PMF Analysis. Proteins separated by monolithic capillary HPLC were deposited on the trypsinized MALDI plate and analyzed for the peptide map by

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Table 1. Identification of Proteins in MCF10A Prefractionated at pH 6.34 for Monolithic RP-HPLC Separation Followed by On-Probe Digestion for MALDI-TOF MS Analysis

collection time (min)	protein name	Accession no.	theoretical MW	theoretical pI	experimental MW	experimental pI	sequence cov (%)	MOWSE score
5.5−6.0	60S ribosomal protein L5	P46777	34448	9.8	34114	6.34	35	2184
	small nuclear ribonucleoprotein associated protein B and B′	P14678	24610	11.2	24583	6.34	34	5355
	glucosamine-fructose-6-phosphate aminotransferase	Q06210	78807	6.7	78816	6.34	43	3712
6.5−7.0	NADH ubiquinone oxidoreductase subunit B14.5b	O95298	14188	9.0	14324	6.34	33	1045
	LIM domain transcription factor LMO4	P61969	17994	8.7	17946	6.34	57	2675
	Ig heavy chain V−III region TEI	P01777	12803	6.9	12808	6.34	57	3625
10.5−11.0	tyrosine-protein kinase ZAP-70	P43403	69873	7.8	69605	6.34	56	1706
	α-1-antichymotrypsin precursor	P01011	47561	5.3	47573	6.34	49	4122
11.5−12.0	ATP synthase B chain (truncated)	P24539	28909	9.4	24893	6.34	22	5253
	succinyl-CoA:3-ketoacid-coenzyme A transferase 1 (truncated)	P55809	56158	7.1	52301	6.34	27	5078
13.0−13.5	protein disulfide isomerase A3 precursor	P30101	56783	6.0	56664	6.34	60	1.59×10^4
13.5−14.0	lamin A/C	P02545	74140	6.6	74501	6.34	59	5.27×10^7
16−16.5	T-complex protein 1, ζ subunit	P40227	58025	6.2	58023	6.34	16	1650
18.0−18.5	keratin, type II cytoskeletal 7 (CK 7)	P08729	51418	5.5	51327	6.34	61	2.85×10^7
	ribosomal protein S6 kinase α 3	P51812	83737	6.4	83618	6.34	60	2.07×10^4
18.5−19.0	stress-70 protein (truncated)	P38646	73681	5.9	68763	6.34	48	1.94×10^4
19.0−19.5	keratin, type I Cytoskeletal 17 (CK 17)	Q04695	48106	5.0	48830	6.34	47	1.30×10^4
	transitional endoplasmic reticulum ATPase	P55072	89323	5.1	89339	6.34	34	2.71×10^4
19.5−20.0	keratin, type II cytoskeletal 6D (CK 6D)	P48667	42469	5.3	42625	6.34	53	4.00×10^5
20.5−21.0	keratin, type I cytoskeletal 19 (CK 19)	P08727	44106	5.0	44012	6.34	76	2.38×10^9
	keratin, type II cytoskeletal 5 (CK 5)	P13647	62248	8.1	63482	6.34	40	2.78×10^5
	mitochondrial intermediate peptidase (truncated)	Q99797	80612	6.7	77016	6.34	53	1.24×10^4
22.5−23.0	L-lactate dehydrogenase A chain	P00338	36689	8.4	36874	6.34	64	1531
	nucleobindin 1 precursor	Q02818	53852	5.1	53669	6.34	49	1849
23.0−23.5	tumor-associated calcium signal transducer 1 precursor	P16422	34921	7.4	35030	6.34	34	9381
	keratin, type II cytoskeletal 6E (CK 6E)	P16870	60224	8.1	60655	6.34	49	1.04×10^6
23.5−24.0	ribosome biogenesis protein Nop10	Q9NPE3	7706	10.0	7787	6.34	70	4138
	thymus-specific serine protease precursor	Q9NQE7	55049	8.3	54989	6.34	25	4973
24.5−25.0	TGF-β receptor type II precursor	P37173	64540	5.6	64508	6.34	48	9147
25.0−25.5	protein kinase C, θ type	Q04759	81866	7.7	82023	6.34	37	4384
	α-actin, cytoplasmic	P68032	42019	5.2	42055	6.34	27	2369
	γ-actin, cytoplasmic	P63260	41793	5.3	41720	6.34	38	8699
	retinol-binding protein I	P09455	15850	5.0	15686	6.34	53	3654
25.5−26.0	β-actin, cytoplasmic	P60710	41737	5.3	41712	6.34	56	1.62×10^4
26.5−27.0	60 kDa heat shock protein (truncated)	P10809	61055	5.7	57973	6.34	63	4.00×10^5
28.5−29.0	pyruvate dehydrogenase protein X component (truncated)	O00330	54123	8.8	47818	6.34	47	4167
29.5−30.0	T-complex protein 1, θ subunit	P50990	59621	5.4	59256	6.34	59	4845

MALDI-TOF MS. The proteins identified in this experiment are summarized in Table 1. All database search results were subjected to manual inspection to consider the following criteria to obtain a confident match: protein hits with the sequence coverage of greater than 20% and the MOWSE score of greater than 10^3. The protein identifications for several proteins were also confirmed by MALDI-MS/MS analysis. A total of 37 unique proteins from ~120 ng of human breast cancer cell lysates prefractionated at pH 6.34 were successfully identified using the monolithic RP-HPLC separation time of 18 min. In this experiment, a 30-s interval was used throughout the experiment. A shorter protein collection time interval of 15 s was also attempted, where the shorter time interval did not improve the total number of proteins identified. This is due to the limited resolution of the 200-μm monolithic column used in these protein separations.

Figure 3 shows a representative MALDI-TOF MS spectrum obtained for one of the proteins analyzed, later identified as stress-70 protein by PMF and sequencing analyses, where several peaks resulting from trypsin autolysis are annotated. To make an assessment for reproducibility of the developed method, the same Rotofor fraction was analyzed in three replicates for comparison for the purpose of protein identifications. Figure 4 includes a zoomed-in spectrum of Figure 3 that was compared with MALDI-TOF MS spectra obtained from two separate LC/MALDI experiments, where all three replicates were obtained at a 15-s collection time interval to result in the identification of the same protein, stress-70 protein. As shown in all three replicate MALDI-TOF MS spectra, many of the tryptic peptides representing the partial coverage of this protein were commonly detected for reliable PMF analysis. The same protein was also analyzed by MALDI-MS/MS, where one of the peptides detected by MALDI MS, LLGQFTLIGIPPAPR (1592.95 Da), was also successfully sequenced to further confirm its presence, as illustrated in Figure 5.

There are a number of distinct advantages noted from this experiment. Although the pH fractionation used in the work required milligrams of sample, the sample loaded on the monolithic capillary column was reduced by 50-fold; thus, other methods using micro-prefractionation can also be interfaced to this tech-

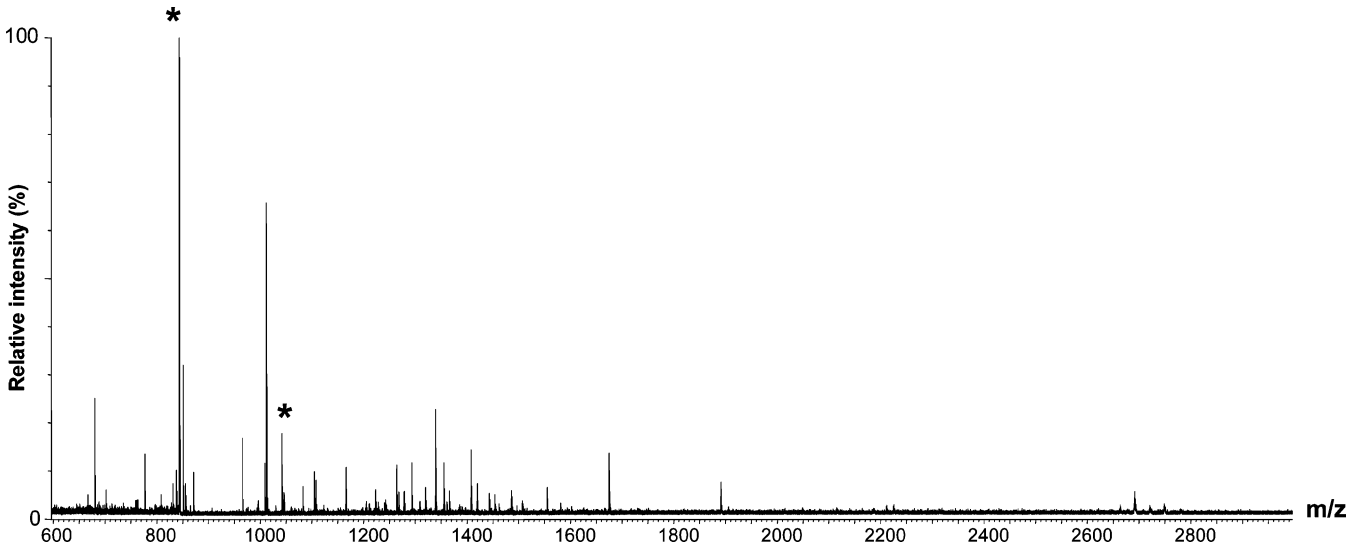

Figure 3. Representative MALDI-TOF MS spectrum, later identified as stress-70 protein (* indicates trypsin autolysis products).

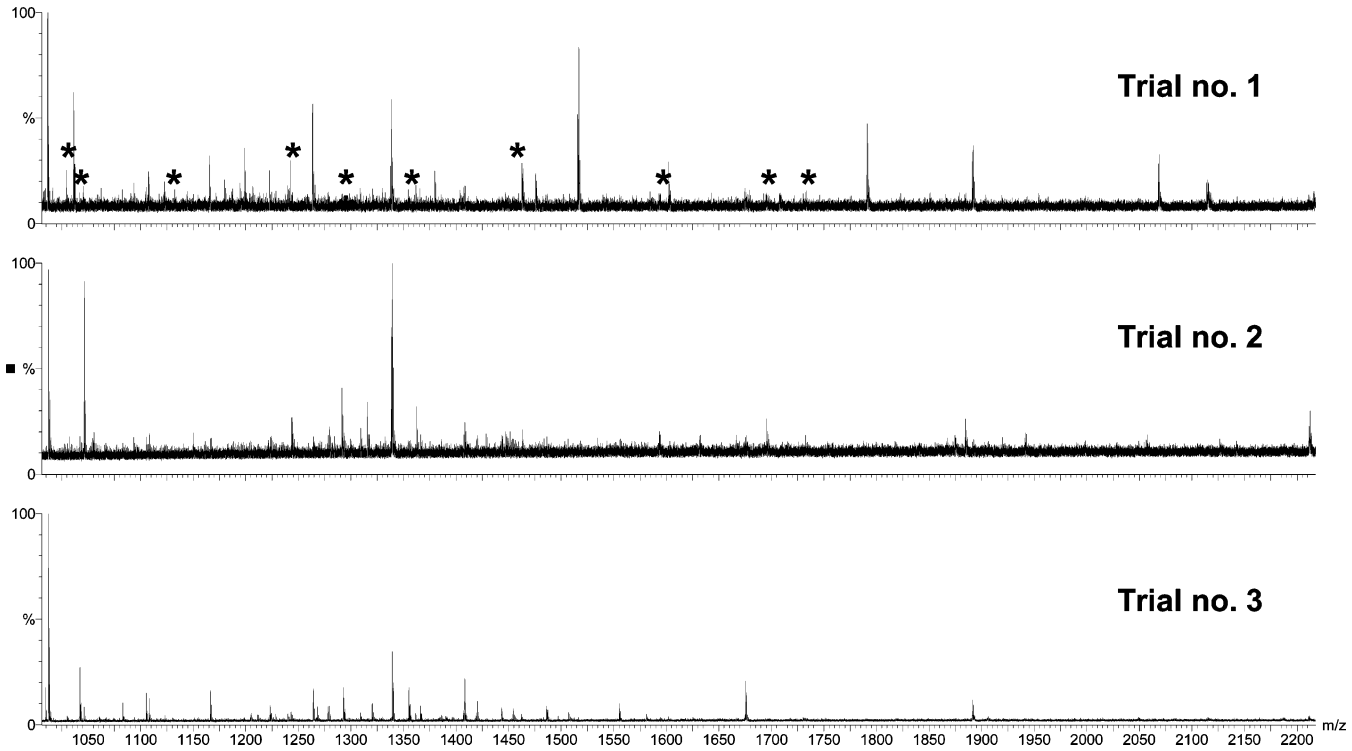

Figure 4. Zoomed-in view of Figure 3 for comparison to MALDI-TOF MS spectra obtained from separate runs (* indicates several tryptic peptides detected in this mass range).

nique for analysis of small amounts of sample. Also, the length of analysis time for analyzing large numbers of samples is an issue where typical in-solution digestion proceeds for hours to overnight, while on-probe digestion completes in 5 min. In addition, the collection of the proteins, enzymatic digestion, and MS analysis are all integrated into one MALDI plate without any sample transfers in order to avoid unnecessary artificial contamination or sample loss. The high recovery provided by a monolithic column and the ability to minimize sample losses contribute to the relatively high sequence coverage obtained by MALDI-TOF MS (Table 1). Also, several injections with DI water prior to the gradient elution wash away impurities contained in the sample, eliminating the need for laborious and costly sample cleanup procedures typically required for MALDI-MS analysis.

The protein identification in this experiment was constrained by the use of the intact MW value obtained by NPS-RP-HPLC/ESI-TOF MS when compared to the theoretical MW of each protein, also shown in Table 1. This table also indicates that slight differences between experimental and theoretical MW values were observed for several proteins. However, these are mitochondrial precursors that lose transit peptides and are truncated.[26] Given this sequence modification, the experimental and theoretical MW values are closely matched based on the identifications obtained by PMF analysis.

In Table 1, one can observe that unique proteins are usually in each fraction. One can also observe that two or more proteins

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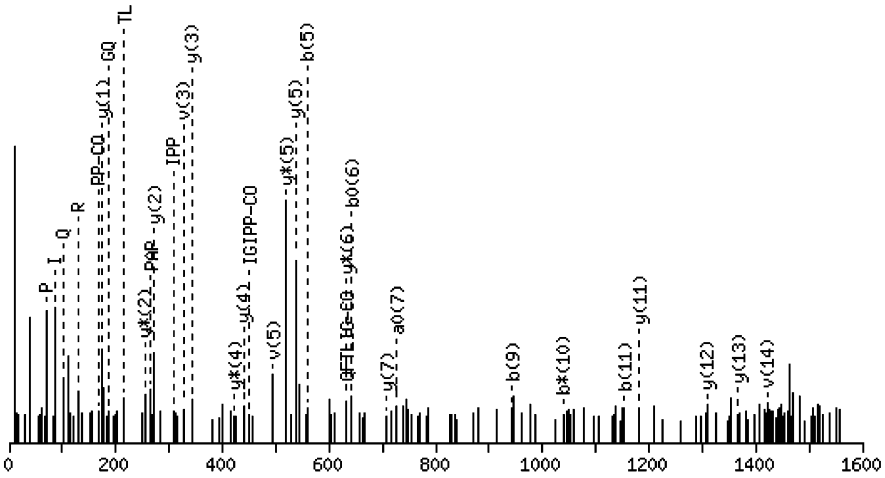

Figure 5. MALDI-MS/MS spectrum for one of the peptides detected in Figure 3, LLGQFTLIGIPPAPR (1592.9500 Da).

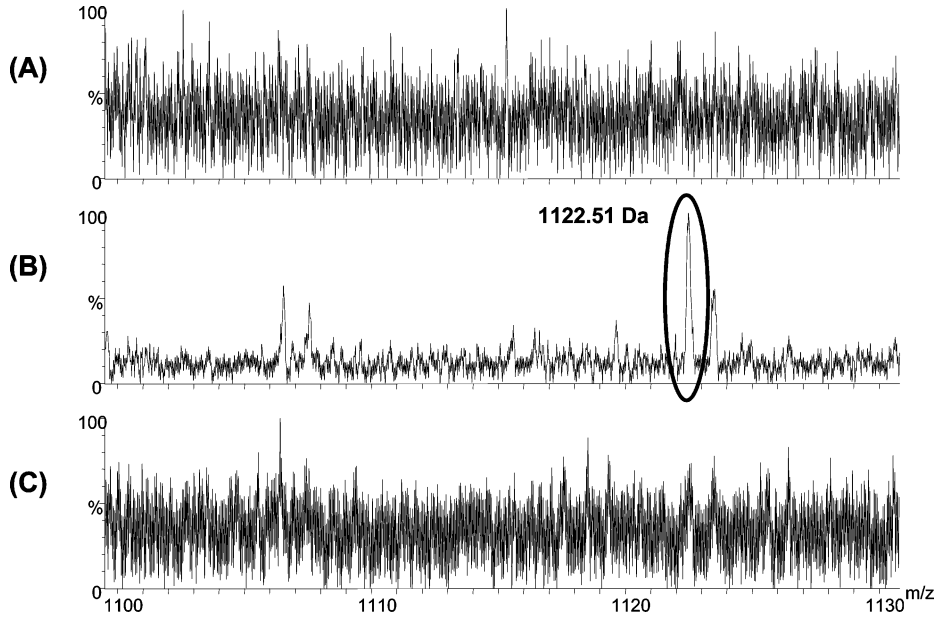

Figure 6. MALDI spectra (zoomed-in views) from monolithic capillary HPLC protein collection time of (A) 5.0−5.5, (B) 5.5−6.0, and (C) 6.0−6.5 min, where the peak at *m/z* of 1122.51 Da corresponds to tryptic peptide of 60S ribosomal protein L5, IEGDMIVCAR.

are often identified from the same spot of the MALDI plate due to the possible coelution of proteins during the monolithic HPLC separation. For example, in the pH range of 6.34, the separation and PMF analysis indicated that there are three unique proteins identified from each of three MALDI spots corresponding to 5.5−6.0, 6.5−7.0, and 20.5−21.0 min of protein collection in 30-s intervals. Figure 6 shows the presence of the two peptides collected at the interval of 5.5−6.0 min, while these were not detected from any of the adjacent spots, implying that proteins were separated with sufficient efficiency to avoid significant overlapping. The database searching unambiguously identifies the two peptides originating from 60S ribosomal protein L5. When three consecutive separation intervals were compared, the presence of one of its tryptic peptides, IEGDMIVCAR (69−78), was only detected from one spot, but not from any of the neighboring spots, suggesting that the protein separation was sufficient for protein identification by this method.

CONCLUSIONS

A method for integrating capillary monolithic RP-HPLC for protein separation with on-probe enzymatic digestion for subsequent MALDI-MS analysis has been demonstrated for obtaining protein identifications for human breast cancer cells in a high-throughput manner. The fast protein separation provided by the monolith-based HPLC combined with off-line interfacing with the MALDI MS is a unique platform for rapid protein identification with improved sequence coverage. The method is simple and robust and also effectively minimizes sample loss by avoiding sample transfers and additional sample cleanup procedures. Tandem MS could also be used with this approach to further confirm the identifications and for structural analysis. Nevertheless, the use of intact protein separation has distinct advantages over total protein digestion of the sample into peptides in that the intact protein method provides protein MW and improved sequence coverage for protein identification.

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