A Proteomic Strategy for Increasing Membrane Protein Identifications with Use of the Agilent High Recovery mRP-C18 Reversed-Phase HPLC Column – A Comprehensive Protein Survey of the HeLa Membrane Proteome

Application

Proteomics

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Abstract

Membrane proteins play pivotal roles in various physiological processes such as signal transduction, molecular transport, and cell-cell interactions and a comprehensive analysis of these proteins is essential to uncovering diagnostic disease biomarkers, therapeutic agents and drug receptor candidates. However, profiling membrane proteins has proven to be particularly challenging because of their hydrophobic nature and low abundance. These obstacles pose major limitations for proteomic techniques such as gel electrophoresis or chromatography-based separation methods. For electrophoretic analyses, many hydrophobic proteins are not readily soluble causing poor gel performance and recoveries, while liquid chromatography (LC) separation techniques may suffer from poor separation characteristics, non-reproducibility and low protein recoveries. To overcome these limitations, new proteomic technologies and strategies are constantly under development. Here, we present a novel and highly robust method for the separation and identification of HeLa cell membrane proteins by an LC-only based separation strategy. Employing optimized reversed phase (RP) conditions and using a uniquely designed reversed-phase column material specifically engineered to enable high protein recoveries, we have identified more than 954 proteins (470 membrane and 337 integral membrane proteins) by HPLC-Chip LC/MS/MS. The optimized reversed-phase (RP) separation and fractionation protocol for intact proteins, combined with TFE in-solution digestion, represents a fast, reliable and reproducible tool for the proteomic characterization of complex hydrophobic protein samples. We have demonstrated that this methodology is a robust alternative to traditional 1D SDS-PAGE after RP fractionation, with the latter requiring much more time and yielding fewer protein identifications.

Introduction

Some of the most important cellular functions are intrinsically tied to biological membranes and a comprehensive analysis of membrane proteins is essential for an in-depth understanding to uncover diagnostic disease biomarkers, therapeutic agents and drug receptor candidates. Membrane proteins play pivitol roles in various physiological processes such as signal transduction, cell-cell contact, the selective transport of molecules and other essential functions. The significance of membrane proteins in drug discovery and drug development is evidenced by the fact that about two-thirds of all drug targets are directed towards these proteins. However, profiling membrane proteins has proven to be particularly challenging because of their low abundance and the difficulties in resolving and identifying them due to their hydrophobic nature.

Traditionally, proteomic analyses of complex protein samples involve the resolution of proteins using 1D or 2D gel electrophoresis (GE) followed by the identification of resolved proteins by mass spectrometry or simply by shotgun proteomics methods. However, the limitations of the electrophoretic separation, such as protein size, extreme pI range and proteins insolubility limit the ability of these methods. Limited dynamic range of detection is also an issue because membrane proteins are typically lower in abundance when compared with soluble proteins. As an alternative approach, reversed-phase high-performance chromatography has been used for resolving membrane proteins and peptides and is used as a means to reduce sample complexity, perhaps prior to GE. But, chromatography of high-molecular mass and hydrophobic proteins also presents its own challenges that often prohibit its use. Column chromatography of hydrophobic proteins can present sample-specific obstacles for researchers and often requires specific expertise in sample solubilization techniques, method development and knowledge of column material types. In addition, RP separations of complex protein samples have suffered from low sample recoveries, poor reproducibility and inadequate resolution. Protein recoveries from RP chromatography typically range from 30%–75% and most column materials do not provide the resolution needed for highly complex sample mixtures, such as those presented by membrane proteins.

In this study, two proteomic sample preparation strategies were evaluated for enabling a comprehensive survey of membrane protein identifications and method robustness with careful consideration given to time consumption and hands-on labor. In the first method (gel-free), HPLC fractions were tryptically digested in-solution and analyzed by 2D HPLC-Chip LC/MS/MS. In the second method (gel-based), the HPLC fractions were further resolved on SDS-PAGE and all gel bands from 9 lanes excised (216 bands in total), tryptically digested, and analyzed by 1D HPLC-Chip LC/MS/MS (Figure 1). Thus, we found that 1D prefractionation alone, with the Agilent mRP-C18 column, sufficiently reduces sample complexity prior to LC/MS/MS analysis. The optimized RP column prefractionation workflow saved considerable sample preparation time and labor, by allowing the omission of an additional SDS-PAGE preparation step, and enabled the identification of more proteins. To date, we have identified 954 proteins (470 membrane and 337 integral membrane) associated with a HeLa membrane sub-fraction. Among the protein identifications are many important cell receptors, identified in Table 1.

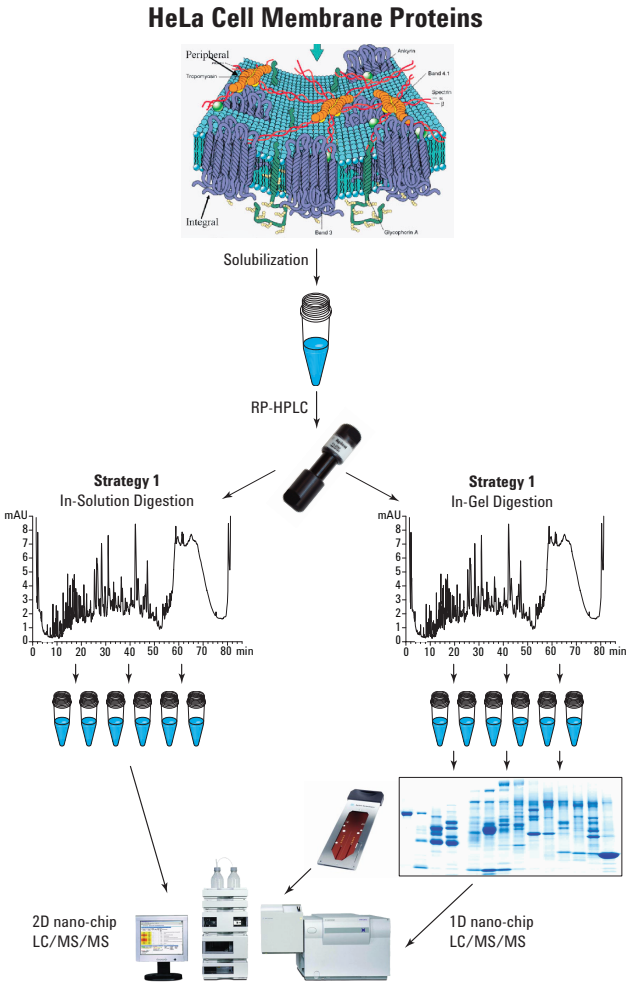


Figure 1. Sample workflows for the identification of HeLa membrane proteins isolated from HeLa cells.

Table 1. Cell Receptor Proteins iidentified by HPLC-Chip LC/MS/MS Using the In-solution Digestion Strategy Shown in Figure 1.

entry_name	accession_number	numPepsUnique	scoreUnique	protein_mw
28 kDa Golgi SNARE protein (Golgi SNAP receptor complex member 1) (28 kDa cis-Golgi SNARE p28) (GOS-28)	O95249	2	21.28	28612.8
40S ribosomal protein SA (p40) (34/67 kDa laminin receptor) (Colon carcinoma laminin-binding protein) (NEM/1CHD4) (Multidrug resistance-associated protein MGr1-Ag)	P08865	4	56.96	32723
Atrial natriuretic peptide receptor A precursor (ANP-A) (ANPRA) (GC-A) (Guanylate cyclase) (EC 4.6.1.2) (NPR-A) (Atrial natriuretic peptide A-type receptor)	P16066	2	21.37	118919.9
Autocrine motility factor receptor, isoform 2 (EC 6.3.2.-) (AMF receptor) (gp78)	Q9UKV5	2	27.85	72996.2
B-cell receptor-associated protein 31 (BCR-associated protein Bap31) (p28 Bap31) (CDM protein) (6C6-AG tumor-associated antigen) (DXS1357E)	P51572	6	81.03	27860.6
cAMP-dependent protein kinase type II-alpha regulatory subunit	P13861	2	27.07	45387.4
Cation-dependent mannose-6-phosphate receptor precursor (CD Man-6-P receptor) (CD-MPR) (46 kDa mannose 6-phosphate receptor) (MPR 46)	P20645	3	45.84	30993.5
Cation-independent mannose-6-phosphate receptor precursor (CI Man-6-P receptor) (CI-MPR) (M6PR) (Insulin-like growth factor II receptor) (300 kDa mannose 6-phosphate receptor) (MPR 300) (MPR300) (CD222 antigen)	P11717	6	59.58	274277.4
CD44 antigen precursor (Phagocytic glycoprotein I) (PGP-1) (HUTCH-I) (Extracellular matrix receptor-III) (ECMR-III) (GP90 lymphocyte homing/adhesion receptor) (Hermes antigen) (Hyaluronate receptor) (Heparan sulfate proteoglycan) (Epi	P16070	4	58.84	81554
CD97 antigen precursor (Leukocyte antigen CD97)	P48960	8	105.81	91841.9
Coxsackievirus and adenovirus receptor precursor (Coxsackievirus B-adenovirus receptor) (hCAR) (CVB3 binding protein)	P78310	8	108.98	40030.1
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 63 kDa subunit precursor (EC 2.4.1.119) (Ribophorin II) (RPN-II) (RIBIIR)	P04844	6	108.43	69284.3
Ephrin type-A receptor 2 precursor (EC 2.7.1.112) (Tyrosine-protein kinase receptor ECK) (Epithelial cell kinase)	P29317	3	33.06	108254.9
Epidermal growth factor receptor precursor (EC 2.7.1.112) (Receptor tyrosine-protein kinase ErbB-1)	P00533	8	115.99	134278.3
Folate receptor alpha precursor (FR-alpha) (Folate receptor 1) (Folate receptor, adult) (Adult folate-binding protein) (FBP) (Ovarian tumor-associated antigen MOv18) (KB cells FBP)	P15328	9	130.41	29819.3
G-protein coupled receptor family C group 5 member C precursor (Retinoic acid induced gene 3 protein) (RAIG-3)	Q9NQ84	2	21.19	48193.5
Inositol 1,4,5-trisphosphate receptor type 1 (Type 1 inositol 1,4,5-trisphosphate receptor) (Type 1 InsP3 receptor) (IP3 receptor isoform 1) (InsP3R1) (IP3R)	Q14643	5	66.26	313946.8
Inositol 1,4,5-trisphosphate receptor type 3 (Type 3 inositol 1,4,5-trisphosphate receptor) (Type 3 InsP3 receptor) (IP3 receptor isoform 3) (InsP3R3)	Q14573	6	79.03	304040.1
Integrin alpha-2 precursor (Platelet membrane glycoprotein Ia) (GPIa) (Collagen receptor) (VLA-2 alpha chain) (CD49b)	P17301	5	78.71	129296.1
Integrin alpha-3 precursor (Galactoprotein B3) (GAPB3) (VLA-3 alpha chain) (CD49c) (FRP-2)	P26006	4	41.21	118698.4
Integrin alpha-5 precursor (Fibronectin receptor alpha subunit) (Integrin alpha-F) (VLA-5) (CD49e)	P08648	4	55.25	114537.1
Integrin alpha-V precursor (Vitronectin receptor alpha subunit) (CD51 antigen)	P06756	7	91.39	116052.5
Integrin beta-1 precursor (Fibronectin receptor beta subunit) (CD29 antigen) (Integrin VLA-4 beta subunit)	P05556	19	235.8	88466
Integrin beta-4 precursor (GP150) (CD104 antigen)	P16144	12	176.15	202152.3
Integrin beta-5 precursor	P18084	3	41.86	88054.9
Keratin, type II cytoskeletal 1 (Cytokeratin 1) (K1) (CK 1) (67 kDa cytokeratin) (Hair alpha protein)	P04264	52	849.2	65886.8
Lamin B receptor (Integral nuclear envelope inner membrane protein) (LMN2R)	Q14739	8	89.47	70703.6
Lysosome membrane protein II (LIMP II) (Scavenger receptor class B, member 2) (85 kDa lysosomal membrane sialoglycoprotein) (LGP85) (CD36 antigen-like 2)	Q14108	2	26.57	54159.3
Membrane associated progesterone receptor component 1 (mPR)	O00264	11	157.74	21540.1
Microsomal signal peptidase 23 kDa subunit (EC 3.4.-.-) (SPase 22 kDa subunit) (SPC22/23) (UNQ1841/PRO3567)	P61009	6	74.61	20313.5
Mitochondrial import receptor subunit TOM22 homolog (Translocase of outer membrane 22 kDa subunit homolog) (hTom22) (1C9-2)	Q9NS69	2	36.46	15521.7
Mitochondrial precursor proteins import receptor (Translocase of outer membrane TOM70)	O94826	13	200.11	67455.2
Neogenin precursor	Q92859	2	25.44	159960.3
Ninein (hNinein)	Q9P2E9	23	327.72	152472.9
Orphan nuclear receptor TR4 (Orphan nuclear receptor TAK1)	P49116	2	20	65414.9
Plexin B2 precursor (MM1)	O15031	23	299.05	205100.3
Polymeric-immunoglobulin receptor precursor (Poly-Ig receptor) (PIGR) [Contains: Secretory component]	P01833	5	77.49	83314
Receptor-type tyrosine-protein phosphatase F precursor (EC 3.1.3.48) (LAR protein) (Leukocyte antigen related)	P10586	3	32.21	211845.8
Receptor-type tyrosine-protein phosphatase S precursor (EC 3.1.3.48) (R-PTP-S) (Protein-tyrosine phosphatase sigma) (R-PTP-sigma)	Q13332	3	32.81	217095.5
Selenoprotein S (VCP-interacting membrane protein) (AD-015) (SBBI8)	Q9BQE4	3	47.63	21116.2
Signal recognition particle receptor alpha subunit (SR-alpha) (Docking protein alpha) (DP-alpha)	P08240	12	147.98	69811.6
Signal recognition particle receptor beta subunit (SR-beta) (Protein APMCF1)	Q9Y5M8	13	192.78	29702.4

Experimental

The high-recovery macroporous reversed-phase C18 column (mRP-C18) for separating proteins [1] is a product from Agilent Technologies (Wilmington, DE). A 4.6-mm × 50-mm mRP-C18 column (part number 5188-5231) was used with an automated Agilent 1100 LC system with a thermostatted autosampler equipped with a 900 µL injection loop, quaternary pump, thermostatted analytical-scale fraction collector and column heating at 80 °C. The reversed-phase separations of HeLa membrane proteins were performed under a set of optimized conditions using a quaternary mobile phase system consisting of multi-segmented and linear elution gradient, with eluent A (0.1% TFA in water, [v/v]), eluent B (0.08% TFA in acetonitrile, [v/v]), eluent C (20% formic acid in acetonitrile [v/v]) and eluent D (2-propanol). The gradient flow rate was 0.75 mL/min and detection was monitored at 280 nm. For consecutive chromatographic runs, a 30-minute post-run comprised of 20.0% eluent B was added to reequilibrate the column.

HeLa Membrane Sample Preparation and Solubilization for HPLC Column Loading

Isolation of membranes from HeLa S3 cells was performed by a modified carbonate fractionation procedure [2, 3]. HeLa S3 cells were grown to 90% confluency in Ham's F12 medium with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate supplemented with 10% fetal bovine serum. After washing with PBS, cells were collected and washed one additional time with PBS and then with 10 mM Tris-HCl, pH-7.0. Cells were centrifuged and the pellet was resuspended in the above Tris buffer with "Complete" protease inhibitors. Cells were allowed to swell for 10 min, and gently homogenized in a tight-fitting Dounce homogenizer (20 strokes). Unbroken cells and debris were removed by centrifugation at 3200 × g for 10 minutes and the cell lysate was diluted with ice-cold 100 mM sodium carbonate, pH-11.5, to a final protein concentration of 0.28 mg/mL. After incubation at 4 °C for 1.5 hr. (slow rotation), lysate was centrifuged at 103,900 × g for 1 hr. at 4 °C. Membrane pellets were rinsed gently with the ice cold water and then with 10 mM Tris-HCl, pH-7.4. Membranes were aliquoted and stored at –80 °C.

Prior to HPLC injection, the membrane fractions required dissolution in formic acid to enable injection onto the column. As needed, 100 µL aliquots (292 µg) of the membrane fraction were dried in a centrifugal vacuum concentrator (Thermo-Savant, Millford, MA), resolubilized in 200 µL of 80% formic acid and briefly sonicated for 30 seconds in a water bath. The samples were then re-dried in the centrifugal vacuum concentrator, resolubilized in 500 µL of 80% formic acid and again briefly sonicated for 30 seconds in a water bath. The final sample concentration was approximately 0.58 µg/µL in 80% formic acid. HPLC injection amounts varied from 200 µL to 500 µL depending on the amount of protein needed for either in-solution digestion or SDS PAGE analysis.

After formic acid solubilization, the samples were separated under high temperature RP conditions using a combination of a multisegmented elution gradient of water (0.1% TFA)/ACN (0.08% TFA) and a linear elution gradient of ACN (20% formic acid)/2-propanol (Table 2). HPLC fraction collection was performed by time, collecting 1.5 minute time slices starting at 1.0 minute and continuing to 70.0 minutes. The fractions were collected into 1.5-mL plastic tubes (part number 5188-5251) at 4 °C. The fractions were then dried in a centrifugal vacuum concentrator (Thermo-Savant, Millford, MA) and stored at –80 °C.

Table 2. Lipid Raft Fractionation Multisegmented Gradient

Flow	0.75 mL/min
Stoptime	86.0 min
Posttime	30.0 min
Column temp.	80.0 °C
Solvent A	Water/0.1% TFA
Solvent B	ACN/0.08% TFA
Solvent C	ACN/20% Formic acid
Solvent D	2-Propanol
Detection UV	280 nm
Pressure limit	250 bar

Gradient

Time (min)	%B	%C	%D
0	20	0	0
14	34	0	0
50	50	0	0
75	100	0	0
77	100	0	0
78	0	100	0
80	0	100	0
83	0	0	100
86	20	0	0

Electrophoretic Analysis

SDS-PAGE analysis was performed on Invitrogen Tris-glycine precast gels (4%–20% acrylamide, 10 wells, 1 mm). Fractions from mRP-C18 RP separations were combined based on UV absorbance at 280 nm and dried in a SpeedVac on low heat. Following resuspension in 2x sample preparation buffer, fractions were heated for 1 min. at 50 °C,

and then loaded onto the gel. Gel-separated proteins were visualized by Coomassie Blue staining using Pierce GelCode Blue (part number 24592).

In-Solution and In-Gel Digestion

In-gel digestion and HPLC-Chip-LC-MS/MS (Gel-Based Method)

Forty-seven mRP-C18 column fractions, collected in 1.5 minute time slices from 0–70 minutes during the separation shown in Figure 2, were combined (based on previous gel pattern results) and loaded into 9 gel lanes. Twenty-four bands from each lane (216 in total) were excised and digested with trypsin using an Agilent in-gel digestion kit (part number 5188-2745). The digested peptides were extracted and proteins identified by LC-MS/MS analysis on an Agilent 1100 HPLC-Chip coupled to the XCT Ultra Ion Trap. The digests were loaded onto a standard RP chip (Agilent, Zorbax 300SB-C18, 5-µm, 0.075-mm id × 43-mm, part number G4240-62001). Elution of peptide fragments was accomplished by RP gradient elution with buffer A; water/3.0% ACN (0.1% formic acid) and buffer B; water/95% ACN (0.1% FA). The XCT Ultra ion trap mass spectrometer was operated in standard scan mode for MS analysis and in ultra scan mode for MS/MS.

The gradient for the RP separations is as follows:

Time (min)	0	2	20	22	22.5	23.5	24
%B	3	10	35	50	95	95	3

In-solution digestion and 2D HPLC-Chip-LC-MS/MS (Gel-Free Method)

Forty-Seven mRP-C18 column fractions were combined into 17 fractions and each combined fraction was dried and digested with trypsin using a TFE digestion protocol [4]. The digested fractions were analyzed with an Agilent 1100 nano-two-dimensional-LC and 1100 MSD trap XCT ultra. The digests were first loaded onto a capillary SCX column (0.25-mm id × 40-mm) and eluted with a series of 2 µL ammonium acetate solution injection with increased concentration.

SCX elution steps (mM):

1	2	3	4	5	6	7	8	9	10	11
20	50	100	150	200	250	350	500	700	1000	2000

The eluted peptides from each salt step were further separated with the same RP chip and gradient as above with the same MS operating conditions. Total analysis time for each combined fraction was about 6 hours.

MS/MS data were searched against the SwissProt Human database (total of 12,015 entries), using Spectrum Mill computer database search algorithm, with the “Calculate Reversed Database Scores” option “on”. The peptide/protein hits were filtered with the “autovalidation” option using the following parameters: minimum score for peptides: +1, 7.0; +2, 8.0 (if SPI larger than 90%, the score was lowered to 7.0); +3, 9.0; +4, 9.0. All peptide matches required a “Forward-Reverse Score” larger than 1.0, and “Rank 1–2 score” larger than 1.0. The protein score was set at a minimum of 15.0. Only fully tryptic peptides were considered, with two missed cleavages allowed.

All MS data from both in-gel and in solution approaches were searched against SwissProt Human database (total 12,015 entries), using Spectrum Mill (Agilent) computer database search algorithm.

Results and Discussion

The Agilent high-recovery macroporous reversed-phase C18 column (mRP-C18) column was used to separate HeLa membrane proteins isolated from a HeLa cell total lysate. Using the Agilent mRP-C18 column and optimized RP chromatographic conditions we collected column fractions and performed a comparative comprehensive analysis of protein identifications by nano-chip LC/MS/MS for protein fractions directly in-solution digested, from RP HPLC, versus an identical set of fractions RP collected and further resolved by SDS-PAGE and in-gel digested.

To evaluate the utility of either strategy (gel-free versus gel-based), an efficient chromatographic separation was established for fraction processing and analysis. Figure 2 is a representative RP chromatogram for the separation of HeLa membrane proteins on a 4.6-mm × 50-mm mRP-C18. Changes to gradient compositions and elution times were systematically performed to optimize this separation, while SDS-PAGE of collected fractions was used to characterize the separation efficiency. The elution conditions and column material enabled a well resolved protein separation and displayed excellent peak shapes ideal for discrete fraction collection. The area of the chromatogram from 0–70 minutes represents the region of highest protein elution. Within this region, we collected 47 RP fractions by time-based autosampling and resolved them on an SDS-PAGE (Figure 3). The electrophoretic analysis of the UV profile details the highly separated proteins and the discrete protein banding patterns.

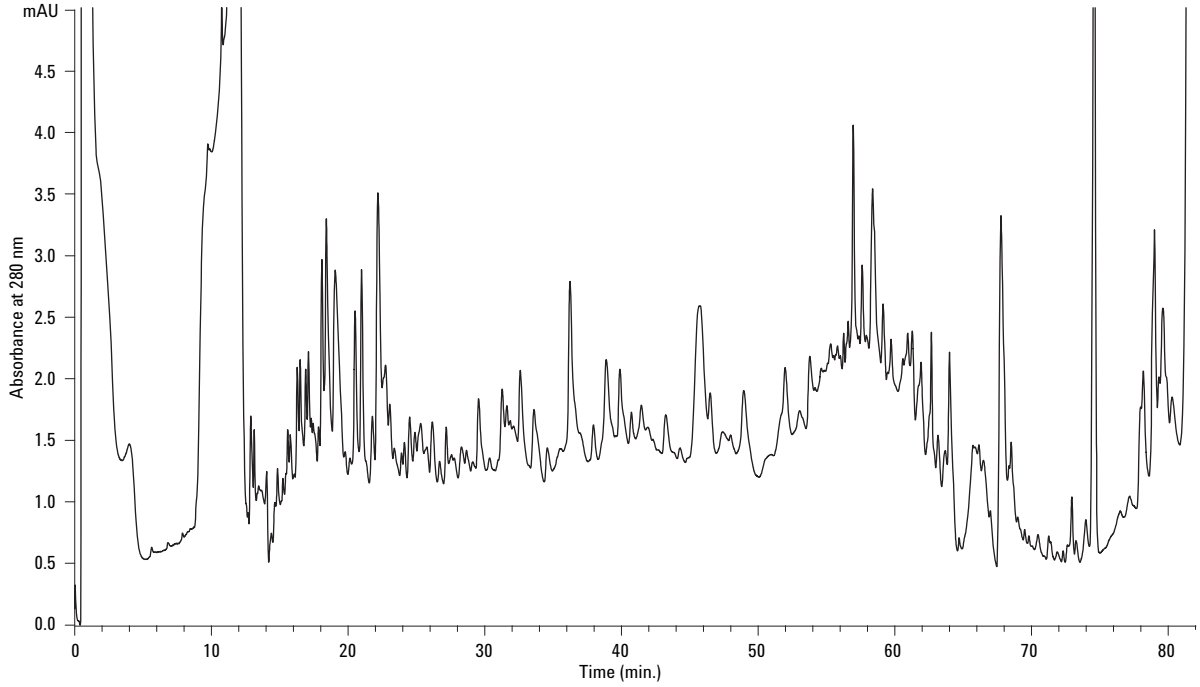
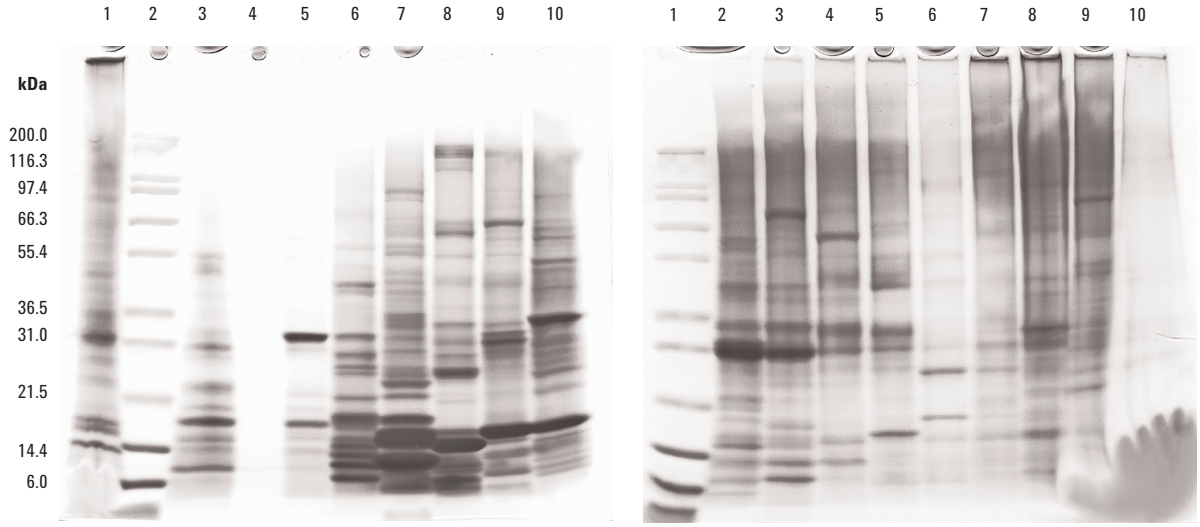


Figure 2. Reversed-phase separation of 300 µg HeLa membrane sub-fraction from a 4.6-mm × 50-mm mRP-C18 column. The region from 0–70 minutes represents the area of highest protein elution as determined by SDS PAGE (shown in Figure 3).



Gel 1

1	HeLa membranes, starting material, 22 µg	
2	Mark12 standards	
3	Fractions	1–4
4	Fractions	5–8
5	Fractions	9–12
6	Fractions	13–16
7	Fractions	17–20
8	Fractions	21–24
9	Fractions	25–28
10	Fractions	29–32

Gel 2

1	Mark12 standards	
2	Fractions	33–36
3	Fractions	37–40
4	Fractions	41–44
5	Fractions	45–48
6	Fractions	49–52
7	Fractions	53–56
8	Fractions	57–60
9	Fractions	61–64
10	Fraction	65–70

Figure 3. SDS PAGE of a HeLa membrane sub-fraction prefracationated by an mRP-C18 chromatography column (see Figure 2). Forty seven fractions were collected at 1.5 minute time intervals from 0-70 minutes and uniquely combined based on previous SDS-PAGE.

Upon establishing an optimized separation protocol and after evaluating SDS PAGE analyses like that shown in Figure 3, RP fractions were futher consolidated and, combined for mass spectrometry analysis by either direct in-solution digestion or by SDS PAGE and gel band digestion. To compare protein identification results from both approaches, MS data acquisition time was kept the same. For the in-solution method, a total of 17 uniquely combined fractions were tryptically digested and analyzed by 2D HPLC-Chip- LC/MS/MS, with a total MS time of approximately 105 hours. For the gel based method, a total of 216 bands form 9 SDS PAGE lanes were analyzed by 1D HPLC-Chip-LC/MS/MS and resulted in a total of 108 hours MS time.

MS results for the gel-free method showed a total of 954 proteins identified with 470 being membrane proteins and 337 of those being integral membrane proteins. Alternatively, results by the gel-based approach gave 688 total proteins with 364 membrane protein identifications and 286 integral membrane identifications. Thus, total protein identifications from the in-solution method were almost 40% greater than the latter method, while the membrane protein identifications were 30% more. In our study, the time saved to omit the SDS PAGE separation and analysis was more than 4 days. Furthermore, the SDS-PAGE method required extensive sampling handling and manipulation effort that was not needed by direct in-solution digestion.

Conclusion

In this study, we showed the advantage of performing mass spectrometric analysis directly following HPLC fractionation (in-solution digestion) of intact membrane proteins without the need for subsequent protein separation by gel-based methods. Employing an HPLC fractionation "only" strategy and utilizing in-solution tryptic digestion and two-dimensional separation of peptides, we identified 954 proteins (470 membrane and 337 integral membrane) associated with a HeLa membrane subfraction. Among these membrane identifications are important pharmaceutical targets, such as EGF receptors, Integrin proteins, TNF receptors, etc. (Table 1).

The Agilent high-recovery mRP-C18 column and optimized chromatographic conditions provided a high degree of resolution and enabled sufficient reduction in sample complexity to profile a HeLa membrane proteome without the need for additional protein separation. The column has previously shown excellent reproducibility and high protein recoveries for hydrophobic sample types, such as lipid rafts [5]. The optimized separation protocol is easy to follow and demonstrates excellent utility for use in proteomic workflows for separating, and fractionating, complex sample mixtures such as membrane proteins.

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