High Recovery HPLC of Lipid Raft Domains with the Agilent mRP-C18 Column – A New Proteomic Strategy for Enabling Membrane Protein Identifications

Application Note

Proteomics

Authors

James Martosella, Nina Zolotarjova, Hongbin Liu,
Pat Perkins, and Susanne Moyer
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

Membrane proteins play a critical role in cellular processes and currently account for ~70% of all known pharmaceutical drug targets. However, despite their biological importance and natural abundance, they remain an under-represented subset of studied proteins within proteomics. Lipid rafts are membrane microdomains that are enriched in cholesterol and glycosphingolipids and it is believed that proteins embedded in the membrane localize either to the clusters of sphigolipids and cholesterol or to the intervening fluid regions of glycerolipids. They are extremely important entities of the plasma membrane and have been implicated in diverse cellular processes, including signal transduction, endocytic events, such as viral entry, and cholesterol trafficking. Yet, while analyses of raft function and composition continues to be of great interest, their study has limitations due to the extremely hydrophobic nature of the raft domains.

A robust proteomic workflow was developed for the identification of membrane proteins from human brain lipid rafts using a novel macroporous reversed-phase column (mRP-C18) and optimized chromatographic conditions. The column conditions and special chromatographic material combine to provide high-protein recoveries, excellent separations and reproducible chromatography. The robust column methodology was used in a multidimensional separation strategy to identify membrane proteins found in lipid rafts. The lipid rafts were fractionated by the mRP-C18 column and further resolved by gel electrophoresis. Unique bands were identified and analyzed by mass spectrometry. Using this strategy we have identified 158 proteins (73 membrane) and 35 integral membrane proteins) from 48 excised gel bands.

Introduction

Some of the most important cellular functions are intrinsically tied to biological membranes. However, due to difficulties in the biochemical purification and structure/function analysis of this protein class, membrane proteins are still much less studied than soluble proteins. Lipid rafts, comprised of enriched region of sphingolipids and cholesterol are inherently more difficult to analyze. Yet, they are extremely important subdomains of the plasma membrane and are implicated in associating with a variety of proteins, especially those involved in cell signaling. Traditionally, proteomic analyses of complex protein samples involve the resolution of proteins using two-dimensional gel electrophoresis (2DGE) followed by the identification of resolved proteins by mass spectrometry (MS) or simply by shotgun proteomics methods, which combines two dimensional liquid chromatography (LC) and

MS. However, by either method, solubility and recovery of proteins remains an obstacle. For 2DGE, many hydrophobic proteins are not solubilized in the nondetergent isoelectric focusing sample buffer and solubilized proteins are prone to precipitation at their isoelectric point. For shotgun proteomic methods, as well as 2DGE, limited dynamic range of detection is also at 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. But, chromatography of high-molecular mass and hydrophobic proteins presents a myriad of challenges that often prohibit its use. Column chromatography can present 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, a robust multidimensional separation strategy for identifying membrane proteins associated with lipid rafts is presented. Using the Agilent mRP-C18 column, we simultaneously separated and delipidated lipid raft proteins and collected fractions for downstream analysis by 1DGE and MS. The mRP-C18 column with use of optimized chromatographic conditions provided high protein recovery and excellent reproducibility. Equally as important the mRP-C18 and optimized chromatographic conditions enables on-column dissociation of the lipids from the membrane proteins and gives researchers the opportunity to independently evaluate the lipid fractions.

Experimental

The high-recovery mRP-C18 for separating proteins 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 an autosampler equipped with a 900-µL injection loop, quaternary pump and column heating at 80 °C. The reversed-phase separations of lipid rafts were performed under a set of optimized conditions using a combined multisegmented 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 re-equilibrate the column.

Sample Preparation and Solubilization of Lipid Rafts and Loading onto the mRP-C18

The lipid raft-enriched fraction was prepared from neurologically normal human brain STG (Superior Temporal Gyrus) by detergent extraction on ice and flotation on sucrose gradient. After homogenization, the sample was incubated in 1% Triton TBS buffer for 30 minutes on ice. A discontinuous sucrose gradient was formed in the centrifuge tubes (Rotor JS - 24.38, Beckman) with the sample in 40% sucrose on the top of 80% sucrose and overlayered with the 35% and 5% sucrose. The gradients were centrifuged for 42 hours at 103,900 × g. During centrifugation, lipid rafts migrated to the low-density region of the gradient due to their high lipid content. Low-density material floating at the 5%–30% interface was collected and centrifuged to pellet the detergent-resistant fraction. Final pellets were suspended in d.H_2O, aliquoted, analyzed for protein concentration by BCA protein assay and stored at –80 °C.

Prior to HPLC injection, the lipid raft fractions required dissolution in formic acid to enable injection onto the column. As needed, 100-µL aliquots of the lipid raft 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 redried 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 1 µg/µL in 80% formic acid. HPLC injection amounts varied from 200 µL–500 µL depending on the amount of protein needed for fraction processing or SDS PAGE analysis.

Lipid Raft HPLC Fractionation

An Agilent 1100 HPLC equipped with a quaternary pump, thermostatted autosampler, thermostatted column compartment, and a thermostatted analytical-scale fraction collector was used for fractionating the samples. Due to the larger volumes required for complete injection of samples, the standard 100-µL needle and sample loop from the autosampler was replaced with a 900-µL needle and metering device (Agilent part number G1363A). HPLC fractions were automatically collected by time 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 for LC-MS/MS and SDS-PAGE analysis.

After lipid raft preparation and subsequent solubilization with formic acid, the lipid rafts 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)/ 2i-Propanol (Table 1). Fraction collection was performed by time, collecting 1-minute time slices starting at 1.0 minute and continuing to 70.0 minutes.

Table 1. Lipid Raft Fractionation Multisegmented Gradient

Flow	0.75 mL/min.			
Stoptime	70.0 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	2i-Propanol			
Detection UV	280 nm			
Pressure limit	250 bar			
Gradient				
	Time (min)	%B	%C	%D
	0	20	0	0
	40	50	0	0
	50	100	0	0
	55	100	0	0
	65	0	100	0
	70	0	0	100
	75	0	0	100
	80	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 minute 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).

Recovery Conditions

To measure protein recovery, lipid rafts were fractionated on the Agilent mRP-C18 column under the gradient conditions shown in Table 1. The entire column effluent from a 200-µg injection was collected. Blank runs were performed in the same manner, however, with the column removed from the flow path. Blanks and column eluates were then dried in a SpeedVac at medium drying temperatures overnight. The proteins were resolubilized in 6M Urea, 1% Triton X-100 and 0.25% acetic acid. Samples were vortexed extensively to solubilize all protein and remove any material adhering to the tube walls. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL). Recovery data for the sample with and without the column installed were used to accurately determine the absolute protein recoveries. Using the same recovery methods, similar results have been obtained by fluorescence detection with use of the EZQ Protein Quantitation kit from Invitrogen/Molecular Probes (part number R33200). Recovery was also determined qualitatively by performing blank runs after each column run. Chromatograms were monitored for the presence of ghost peaks after runs to establish the presence of any protein and lipid carryover.

1D-LC-MS/MS

Gel bands were digested with trypsin using an Agilent in-gel digestion kit (part number 5188-2745). The digested peptides were extracted and proteins were identified by LC-MS/MS analysis on an Agilent 1100 nano-LC and 1100 MSD trap XCT. The digests were loaded onto a capillary 300SB-C18 RP column (Agilent, 0.075 mm id × 150 mm, part number 5065-9911). 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/90% ACN (0.1% FA). The XCT ion trap mass spectrometer was operated in standard scan mode for MS analysis and in ultrascan mode for MS/MS. Forty-eight gel bands were analyzed and MS data were searched against SwissProt Human database (total 12015 entries), using Spectrum Mill (Agilent) computer database search algorithm.

The gradient for the RP separations is as follows:

Time (min)	0	5	40	50	60	62
%B	5	12	35	65	100	100

Results and Discussion

The majority of currently used techniques to analyze complex membrane-protein samples employ 2DGE coupled with MS or tandem LC shotgun MS methods. However, these techniques are limited due to poor protein solubilities, low protein loads, and losses associated with poor protein recoveries.

The Agilent mRP-C18 column and optimized chromatographic conditions provide desirable separation characteristics which demonstrate excellent utility for use in proteomic workflows and offer several advantages over standard RP columns and systems for separating, and fractionating, complex sample mixtures such as lipid rafts.

Figure 1 is representative of a RP separation of human brain lipid rafts on a 4.6-mm × 50-mm mRP-C18. The area of the chromatogram from 0–48 minutes represents the region of highest protein elution, while the elution profile past this region consists mainly of lipids. Gradient compositions, elution times, and elevated temperature, combined with the novel macroporous column material enabled on-column lipid dissociation and minimized irreversible binding effects from lipids, cholesterol and proteins. The lipid elution profile shown in Figure 1 was analyzed by ESI LC-MS/MS (data not shown) and contains significant amounts of phospholipids and sphingomyelins. On-column delipidation allows researchers to minimize protein losses commonly associated with extraction and precipitation methods.

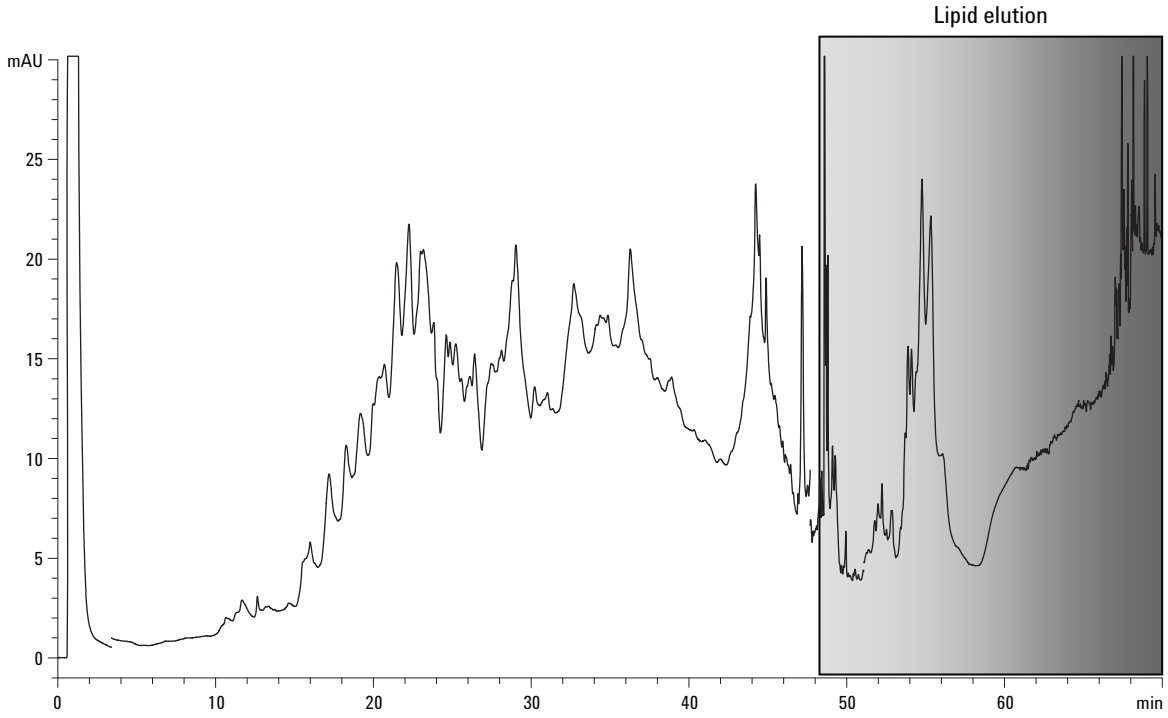

Figure 1. Reversed-phase separation of 500-µg human brain lipid rafts from a 4.6-mm x 50-mm mRP-C18 column. The shaded region past 48 minutes represents the area of highest lipid elution (Chromatographic run elutes to 80 minutes, however, the region past 70 minutes is significantly off-scale due to the i-propanol UV trace and extended lipid elution.)

Using the combination of chromatography with electrophoretic analysis can enable a more informative approach to define the efficiency of an HPLC separation. We collected fractions from an mRP-C18 lipid raft separation and evaluated the chromatographic efficiency on an SDS PAGE (Figure 2). The electrophoretic analysis of the separation detailed excellent protein resolution from 0–48 minutes and showed later eluting lipids past the 48.0 minute area which would not enter the gel lanes (data not shown).

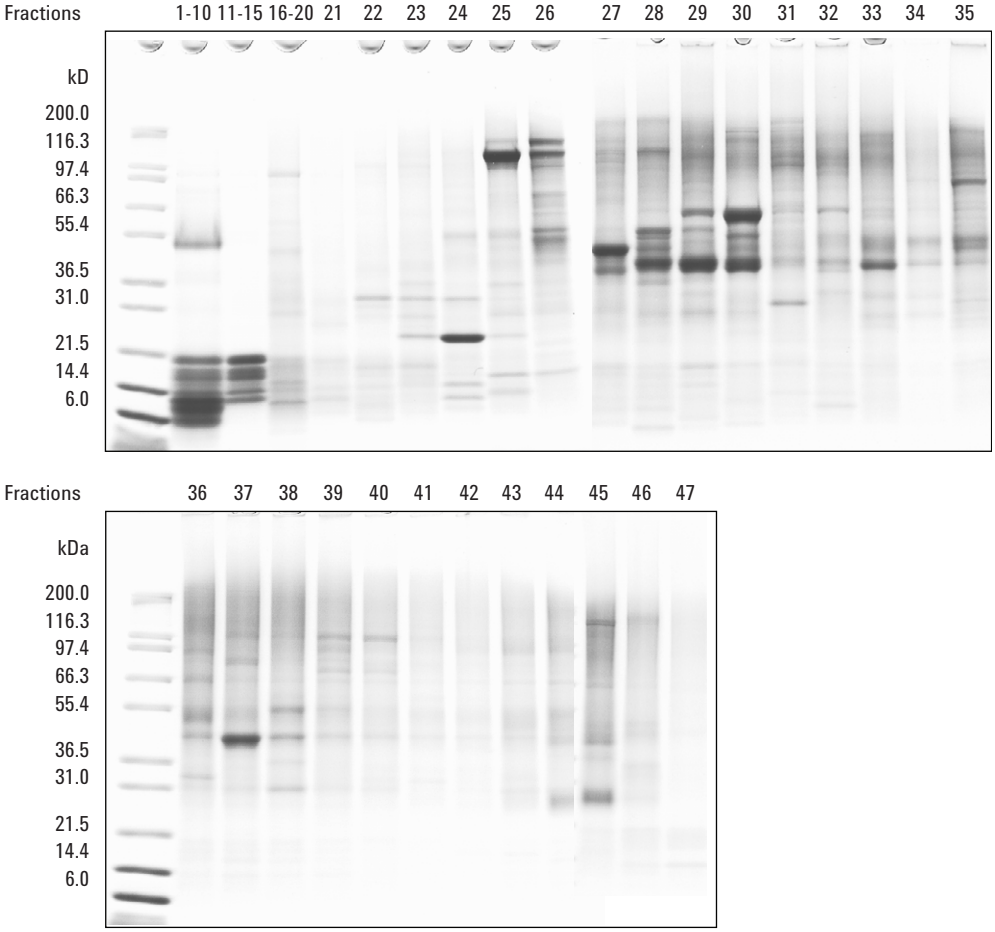

Figure 2. 1D SDS PAGE of the lipid raft separation shown in Figure 1. Fractions were combined based on earlier SDS PAGE. A total of 48 gel bands were excised for LC/MS analysis.

High protein recovery is a critical parameter for useful protein prefractionation strategies and enables reproducible chromatography. This is critical for the validation of biomarkers by allowing for a direct quantitation between control and diseased samples and also is important for multiple sample processing and run-to-run reproducibility. Recovery data for the lipid rafts samples is shown in Table 2.

Table 2. Recovery of Lipid Raft Proteins from the Agilent mRP-C18 Column as Determined by BCA Assay and Fluorescence Detection

Protein conc. No column (µg)	Protein conc. mRP-C18 recovery (µg)	% recovery (N = 4)
112	130	113.8 ± 10.9

Starting protein concentrations were measured with column blanks (no column in the flow path). Column runs were normalized against the blank runs under the same elution conditions. As shown in the table, the mRP-C18 column and optimized HPLC operational conditions provided high recovery of injected sample. Two methods of analysis gave complimentary high recovery data at approximately 114% to the normalized sample. Recovery measurements after mRP-C18 consistently yielded higher protein concentrations than the blank runs which had the column removed from the flow path. We believe the increase in recovered protein was the result of the on-column lipid dissociation thus making the delipidated raft proteins more accessible for the assay.

Pre- and post-column hydrophobic peptide separations were performed to evaluate the column surface after multiple lipid raft separations. Standard C18 reversed-phase stationary phases will irreversibly bind hydrophobic proteins and peptides and cause shifts in retention behavior and losses in total peak area. The changes are good indicators for column efficiency and thus can help in determining protein recovery. An initial peptide separation was established on a new mRP-C18 column and the retention times compared to a post-peptide separation after multiple lipid raft injections. As shown in Figure 3, the retention shifts are nominal and peak areas remain unchanged. In both pre- and post-column injections, peptide peaks have retained their retention times within accepted chromatographic error and show no indications of change due to surface-bound proteins.

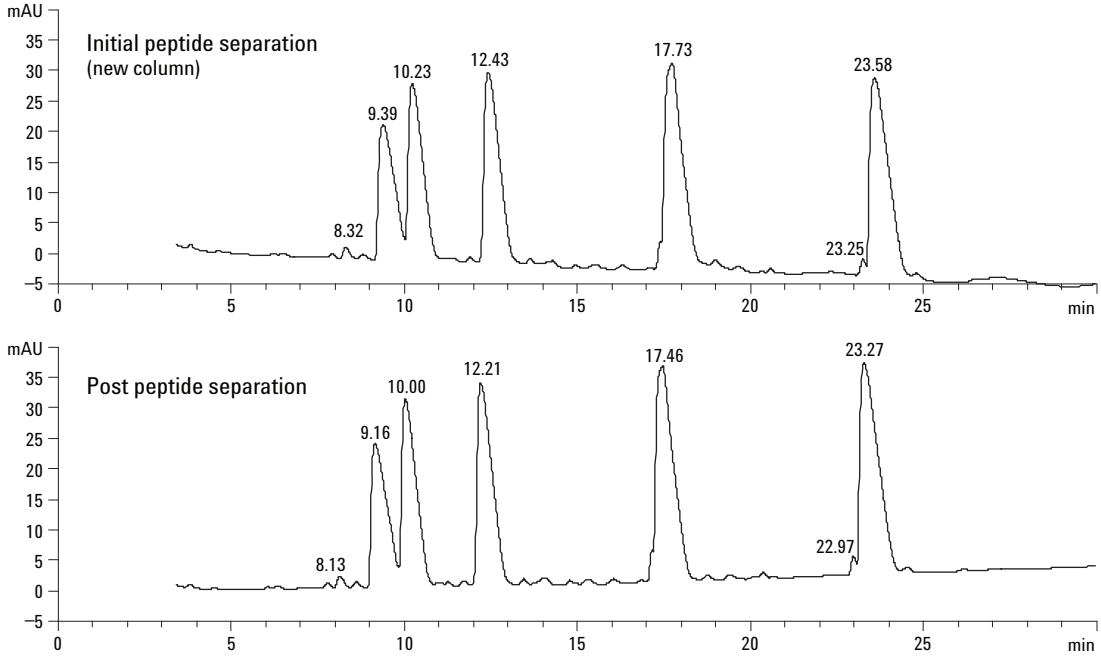

Figure 3. Pre-and post-column peptide separations for characterizing the mRP-C18 surface. Nominal retention shifts and consistent peak shapes indicate that the mRP-C18 column surface has remained unchanged after four consecutive 221-µg lipid raft injections.

Protein carryover displayed as chromatographic peak ghosting is a qualitative indicator for poor column recovery. With poor column recoveries, blank injection column elutions will slowly bleed proteins and require either subsequent blank runs or column wash regimes to establish a steady baseline. As a final gauge to measure protein recovery, consecutive lipid-raft injections were run followed by a subsequent blank injection (blank run) to qualitatively determine the presence of protein carryover. As shown in Figure 4, pre- and post-column blank injections are the same and show no indication of ghosting. Consecutive chromatographic runs prior to the blank injections are displayed in Figure 5. High protein recovery is attributed to both the stabilized macroporous C18 column material and the optimized elution conditions.

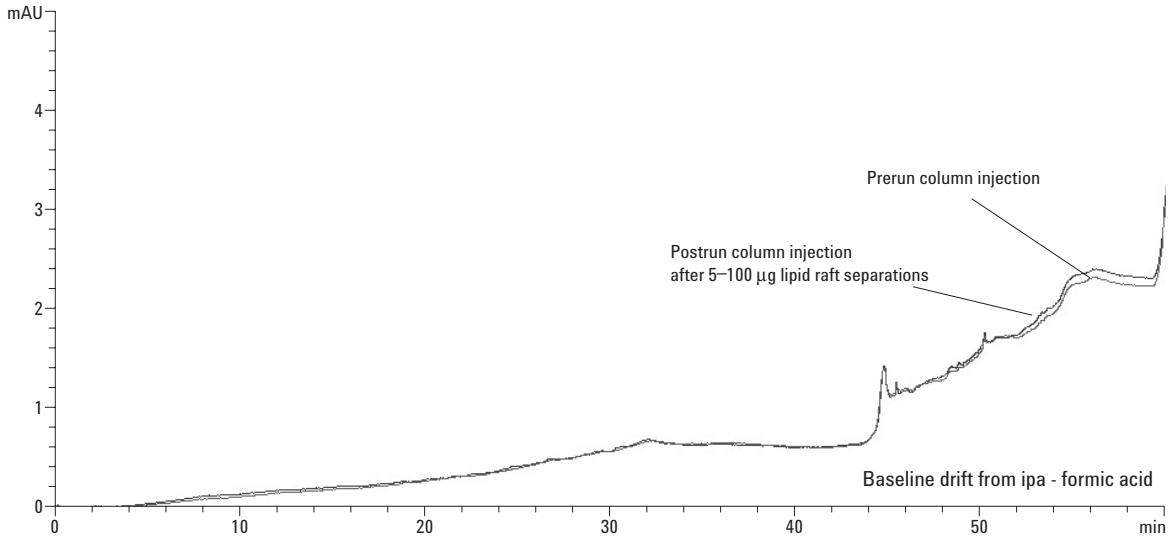

Figure 4. mRP-C18 new column blank and blank column injection after 5–100 µg lipid raft runs to qualitatively detail recovery. The absence of peak ghosting indicates high protein recovery and enables continual column use for multiple sample processing.

Reproducibility would provide a measure of confidence to compare and validate potential protein membrane biomarkers identified in lipid rafts. Chromatographic reproducibility was examined using the preferred quaternary elution conditions and elevated temperature. Separations of lipid rafts from three injections were compared and are shown in Figure 5. The RP separations are reproducible and show no changes from run-to-run in retention times, apparent selectivity, or band widths.

The mRP-C18 column was used to collect a total of 70 reversed-phase fractions using the optimized chromatographic conditions presented. After visualization (Figure 2) of the 1D SDS PAGE, 48 bands were chosen and excised, in-gel digested and subsequently analyzed by 1D LC-MS/MS. The mRP-C18 prefractionation strategy combined with the subsequent resolution on a 1D PAGE enabled the identification of 158 proteins associated with the lipid raft. Of these identified, 73 proteins were identified as membrane proteins with 35 of those being integral membrane proteins. Table 3 shows the identifications for the integral membrane proteins as identified by GO Miner database.

Using the mRP-C18 column to prefractionate human lipid rafts, we suggest a proteomic strategy for identifying membrane proteins. This robust and highly reproducible workflow has demonstrated utility for increasing membrane protein identifications from a highly complex and hydrophobic lipid raft sample and holds enormous potential for identifying membrane protein biomarkers in clinically relevant sample types. In addition, on-column delipidation offers researchers the ability to separate and analyze the lipid portion of the sample, separate from the protein fractions and thus allows for a more complete lipid analysis. Lipid separation by the mRP-C18 column and the optimized elution conditions presented, eliminates protein losses which may occur with more commonly associated techniques, such as solvent extraction.

The mRP-C18 reversed-phase separation of lipid rafts permitted total protein recoveries approaching quantitative, displayed-enhanced peak resolution, showed excellent reproducibility, and enabled on-column lipid dissociation. The combination of the Agilent mRP-C18 RP column and optimized chromatographic conditions, with the subsequent gel separation, enables researchers to reduce sample complexity and thus permit a higher certainty for increasing protein identifications.

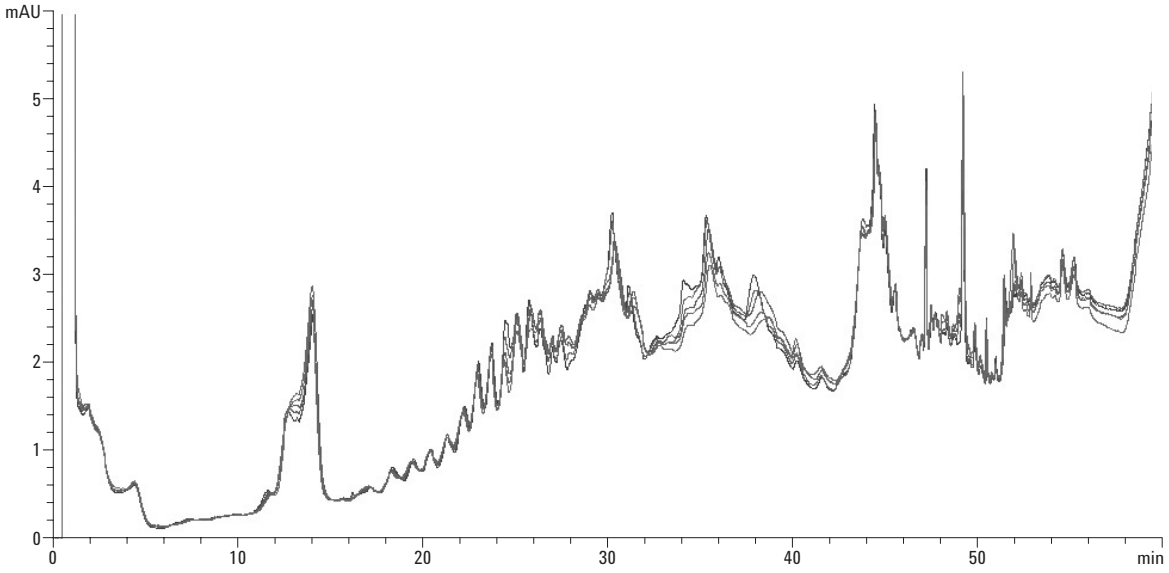

Figure 5. Reproducibility of the mRP-C18 column as shown in five consecutive 100 µg lipid raft injections. Column blanks were performed between each run, shown in Figure 4, and indicate no apparent protein carryover .

Table 3. Thirty-Five Human Brain Lipid Raft Associated Integral Membrane Protein Identifications from 48 1D SDS PAGE Bands

Protein name	Accession number	Unique protein score	Protein MW	Spectra	Mean intensity
Microsomal glutathione S-transferase 3 (EC 2.5.1.18) (Microsomal GST-3) (Microsomal GST-III)	O14880	24.07	16516.4	2	58100000
Flotillin-1	O75955	46.33	47355.5	4	19600000
Protein C8orf2 (UNQ2441/PRO9924/PRO5003)	O94905	20.4	37839.8	2	26800000
Thy-1 membrane glycoprotein precursor (Thy-1 antigen) (CDw90) (CD90 antigen)	P04216	54.56	17934.8	61	1150000000
Sodium/potassium-transporting ATPase beta-1 chain (Sodium/potassium-dependent ATPase beta-1 subunit)	P05026	71.81	35061.5	10	426000000
Dihydrolipoyl dehydrogenase, mitochondrial precursor (EC 1.8.1.4) (Dihydrolipoamide dehydrogenase) (Glycine cleavage system L protein)	P09622	31.15	54150.5	2	90100000
Cytochrome c oxidase polypeptide VIc precursor (EC 1.9.3.1)	P09669	26.54	8781.5	13	303000000
ADP,ATP carrier protein, heart/skeletal muscle isoform T1 (ADP/ATP translocase 1) (Adenine nucleotide translocator 1) (ANT 1) (Solute carrier family 25, member 4)	P12235	39.76	32933.5	3	16800000
Cytochrome c oxidase subunit IV isoform 1, mitochondrial precursor (EC 1.9.3.1) (COX IV-1) (Cytochrome c oxidase polypeptide IV)	P13073	114.67	19576.8	66	77100000
Neural cell adhesion molecule 1, 120 kDa isoform precursor (N-CAM 120) (NCAM-120) (CD56 antigen)	P13592	24.62	83770.5	2	53000000
Neural-cadherin precursor (N-cadherin) (Cadherin-2)	P19022	21.63	99851.9	2	8740000
Vacuolar ATP synthase subunit B, brain isoform (EC 3.6.3.14) (V-ATPase B2 subunit) (Vacuolar proton pump B isoform 2) (Endomembrane proton pump 58 kDa subunit) (HO57)	P21281	355.35	56501	118	166000000
Vacuolar ATP synthase subunit C (EC 3.6.3.14) (V-ATPase C subunit) (Vacuolar proton pump C subunit)	P21283	161.39	43941.8	23	263000000
Voltage-dependent anion-selective channel protein 1 (VDAC-1) (hVDAC1) (Outer mitochondrial membrane protein porin 1) (Plasmalemmal porin) (Porin 31HL) (Porin 31HM)	P21796	207.7	30641.5	103	51100000
Oligodendrocyte-myelin glycoprotein precursor	P23515	49.85	49608.2	4	146000000
ATP synthase B chain, mitochondrial precursor (EC 3.6.3.14)	P24539	81.95	28908.8	12	139000000
ATP synthase alpha chain, mitochondrial precursor (EC 3.6.3.14)	P25705	182.46	59750.9	14	131000000
Prohibitin	P35232	41.63	29804.2	4	14200000
ATP synthase gamma chain, mitochondrial precursor (EC 3.6.3.14)	P36542	17.39	32996.2	2	37900000
Vacuolar ATP synthase subunit E (EC 3.6.3.14) (V-ATPase E subunit) (Vacuolar proton pump E subunit) (V-ATPase 31 kDa subunit) (P31)	P36543	203.63	26145.5	85	171000000
Vacuolar ATP synthase catalytic subunit A, ubiquitous isoform (EC 3.6.3.14) (V-ATPase A subunit 1) (Vacuolar proton pump alpha subunit 1) (V-ATPase 69 kDa subunit 1) (Isoform VA68)	P38606	17.58	68304.5	2	46900000
Voltage-dependent anion-selective channel protein 2 (VDAC-2) (hVDAC2) (Outer mitochondrial membrane protein porin 2)	P45880	42.79	38092.9	8	37300000
Dihydropyridine-sensitive L-type, calcium channel alpha-2/ delta subunits precursor	P54289	47.68	123184	4	34000000
Vacuolar ATP synthase subunit d (EC 3.6.3.14) (V-ATPase d subunit) (Vacuolar proton pump d subunit) (V-ATPase AC39 subunit) (V-ATPase 40 kDa accessory protein) (P39) (32 kDa accessory protein)	P61421	46.33	40329.3	3	99100000
Contactin associated protein 1 precursor (Caspr) (Caspr1) (Neurexin 4) (Neurexin IV) (p190)	P78357	318.22	156267.5	42	152000000

Table 3. Thirty-Five Human Brain Lipid Raft Associated Integral Membrane Protein Identifications from 48 1D SDS PAGE Bands (continued)

Protein name	Accession number	Unique protein score	Protein MW	Spectra	Mean intensity
Contactin 2 precursor (Axonin-1) (Axonal glycoprotein TAG-1) (Transient axonal glycoprotein 1) (TAX-1)	Q02246	46.22	113393.9	4	118000000
Mitochondrial 2-oxoglutarate/malate carrier protein (OGCP)	Q02978	25.01	33930.7	2	28900000
Opioid binding protein/cell adhesion molecule precursor (OBCAM) (Opioid-binding cell adhesion molecule) (OPCML)	Q14982	30.8	38007.8	4	74600000
Myelin-oligodendrocyte glycoprotein precursor	Q16653	54.63	28179.2	8	28500000
Homer protein homolog 1	Q86YM7	84.34	40277	6	72500000
Vacuolar proton translocating ATPase 116 kDa subunit a isoform 1 (V-ATPase 116-kDa isoform a1) (Clathrin-coated vesicle/synaptic vesicle proton pump 116 kDa subunit) (Vacuolar proton pump subunit 1) (Vacuolar adenosine triphosphatase	Q93050	46.42	96413.3	5	28500000
Toll-interacting protein	Q9H0E2	25.85	30282	2	18800000
Pleckstrin homology domain-containing protein family B member 1 (Pleckstrin homology domain retinal protein 1) (PH domain containing protein in retina 1) (PHRET1)	Q9UF11	27.3	27186.1	2	16600000
Bassoon protein (Zinc-finger protein 231)	Q9UPA5	26.43	416370.1	2	24100000
Vacuolar ATP synthase subunit D (EC 3.6.3.14) (V-ATPase D subunit) (Vacuolar proton pump D subunit) (V-ATPase 28 kDa accessory protein)	Q9Y5K8	148.31	28263	24	105000000

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