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Optimization of Protein Solubilization for the Analysis of the CD14 Human Monocyte Membrane Proteome Using LC-MS/MS

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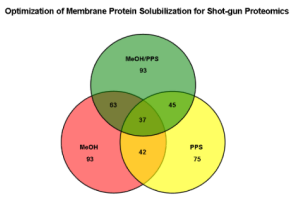
Abstract



Proteomic profiling of membrane proteins is of vital importance in the search for disease biomarkers and drug development. However, the slow pace in this field has resulted mainly from the difficulty to analyze membrane proteins by mass spectrometry (MS). The objective of this investigation was to explore and optimize solubilization of membrane proteins for shotgun membrane proteomics of the CD14 human monocytes by examining different systems that rely on: *i)* an organic solvent (methanol) *ii)* an acid-labile detergent 3-[3-(1,1-bisalkyloxyethyl)pyridin-1-yl]propane-1-sulfonate) (PPS), *iii)* a combination of both agents (methanol + PPS). Solubilization efficiency of different buffers was first compared using bacteriorhodopsin as a model membrane protein. Selected approaches were then applied on a membrane subproteome isolated from a highly enriched human monocyte population that was ~98% positive for CD14 expression by FACS analysis. A methanol-based buffer yielded 194 proteins of which 93 (48%) were mapped as integral membrane proteins. The combination of methanol and acid-cleavable detergent gave similar results; 203 identified proteins of which 93 (46 %) were mapped integral membrane proteins. However, employing PPS a total of 216 proteins of which 75 (35 %) were mapped

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CONFLICT OF INTEREST STATEMENT
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integral membrane proteins. These results indicate that methanol unaided or in combination with PPS yielded significantly higher membrane protein identification/enrichment than the PPS alone.

Keywords

CD14 monocyte; Membrane proteins; Solubilization; Methanol; Detergents; LC-MS/MS

1. Introduction

Membrane proteins play an important role in the structural and functional organization of the cell and cellular organelles. They are important bio-effectors in many of physiological and pathological processes regulating dynamic pathways of eukaryotic mammalian cells [1]. Physiological processes involving signal transduction and their subsequent integration into distinct cellular pathways result in specific cellular responses (i.e. assembly of mitochondrial cytochrome c-oxidase [2]. Pathological processes are exemplified in dysregulation of programmed cell death through extrinsic apoptotic pathway (i.e. cancer) [3]. It has been estimated that 65% of all contemporary pharmaceuticals target membrane proteins while ~ 21 % of all open reading frames from sequenced human genome encode alpha-helical integral membrane proteins [4,5]. Certain membrane proteins have important clinical roles. The status of several membrane proteins or their corresponding genes is key determinants in molecular oncology. They can be used as prognostic (an estimate of likely disease outcome or aggressiveness) or predictive (aid in treatment assignment) [6]. Relevant examples include: ErBb2/HER2 in breast cancer and EGFR in lung and colon cancer [6]. The critical role of membrane proteins in cellular biology and pathology along with their role in therapeutic modulation underscores the importance of a mass spectrometry (MS)-based proteomics in large-scale profiling of human membrane proteins [1].

Monocytes along with lymphocytes represent a category of leukocytes called agranulocytes, and are characterized by the absence of granules in their cytoplasm [7]. Human monocytes develop from myelo-monocytic stem cells in the bone marrow [8]. When released into the blood stream, they differentiate into two major subpopulations: the classical CD14 monocytes and the pro-inflammatory CD14/CD16 monocytes[9]. Monocytes that migrate further into tissues mature into macrophages [7]. Human circulating monocytes constitute ~ 5 % of the leukocytes present in peripheral blood. They play significant role in several highly regulated biological processes including: host defense, inflammation, and tumor surveillance [10,11]. The regulation of these processes is facilitated by distinct cellular mechanisms including: phagocytosis, pinocytosis, chemotaxis and the release of cytokines[10]. At the molecular level these processes are not fully understood. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analyses of the human monocyte proteome have been conducted, resulting in a detailed characterization of the monocyte cytosolic proteome [12,13]. While analyses targeting lipid rafts of monocytic cell lines have been previously reported [14,15], targeted shotgun analysis of the human circulating CD14 monocytes and corresponding membrane proteins should enable better understanding of their biological function, since cell line conditions do not always resemble those *in vivo* [16].

The ability to characterize membrane proteins using MS-based proteomics has lagged behind that of soluble proteins, mainly due to insolubility of integral membrane proteins in natural aqueous buffers [1,17]. Based on their widespread use in membrane biochemistry, gel-based approaches utilizing chaotropes and detergents for membrane protein solubilization prior to 1D or 2D-PAGE have been coupled with MS and employed in membrane proteomics [18]. Low resolution of 1D-PAGE and typically poor yield of

membrane proteins by 2D-PAGE , resulted in development of gel-free shotgun methods employing chaotropes (urea, guanidine) or classic detergents (i.e. SDS, CHAPS, Triton X-100) to solubilize membrane proteins [1]. However, chaotropes impede proteolysis while detergents interfere with separation and MS ionization efficiency of peptides and proteins [19,20]. Although significant advances have been achieved in removal of detergents and chaotropes [21,22], these steps require extensive manipulations and result in sample losses, which are particularly critical when starting with limited amounts of membrane proteins. To avoid negative effects caused by chaotropes and classical detergents, alternative chaotrope/detergent-free shotgun approaches have been developed and employed for the MS analysis of membrane proteins [23-27]. Recently developed mass spectrometry-compatible surfactants have been increasingly used [28-30] in MS-based proteomics and proposed for shot-gun proteomics of membrane proteins [31-35]. Improved MS compatibility of these surfactants is based on their hydrolysis at low pH after digestion that results in minimal interference with downstream reversed-phase peptide separations and MS analysis[29].

Previously, we demonstrated the capability of 60% (v/v) buffered methanol (CH_3OH) vs. non-solubilized membrane preparation to enrich integral membrane proteins for shot-gun proteomics by improving solubilization and digestion of complex membrane protein mixtures isolated from prokaryotic cells [36]. Subsequently we extended this approach, to eukaryotic mammalian systems [37]. The advantage of methanol-based vs. gel-based approach has been also demonstrated [38] and compared to other commonly used approaches[39-42]

The objective of this investigation was to optimize membrane protein solubilization and proteolysis prior to shotgun LC/MS analysis of the human monocyte membrane proteome. To accomplish this goal we compared different approaches that rely on *i)* 60% methanol in 50 mM ammonium bicarbonate (*v/v*), *ii)* 0.1% {3-[3-(1,1-bisalkyloxyethyl)pyridin-1-yl]propane-1-sulfonate (PPS) in 50 mM ammonium bicarbonate (*v/v*), *iii)* a combination of both, 60% methanol and 0.1 % PPS in 50 mM ammonium bicarbonate (*v/v*) and, *iv)* 8 M urea . These approaches were first tested on a single integral membrane protein to asses the solubilizing efficiency and completeness of tryptic digestion and then used for the solubilization and digestion of crude membrane preparation from a highly enriched human monocyte population. Our results demonstrate that methanol alone, or in combination with PPS, is more effective than PPS for the shotgun analysis of integral membrane proteins.

2. Materials and methods

2.1 Reagents

Bacteriorhodopsin from *Halobacterium halobium* (lyophilized purple membrane preparation) was obtained from Sigma (St. Louis, MO, USA). HPLC-grade methanol (CH_3OH) was from EM Science (Darmstadt, Germany). Acid cleavable detergent: 3-[3-(1,1-bisalkyloxyethyl)pyridin-1-yl]propane-1-sulfonate (PPS) was purchased from Protein Discovery Inc. (Knoxville, TN). Ammonium bicarbonate (NH_4HCO_3), phenylmethylsulfonyl fluoride (PMSF), urea and formic acid (HCOOH), and 2-iodoacetamide (IAA) were obtained from Sigma (St. Louis, MO) Tris[2-carboxyethyl] phosphine (TCEP) Bond-Breaker ™ was from Pierce (Rockford, IL Fused-silica capillaries were acquired from Polymicro Technologies (Phoenix, AZ). All chemicals used were A.C.S. grade or higher, and all solvents used were HPLC grade or higher. Sequencing grade trypsin was obtained from Promega (Madison, WI). All solutions were prepared using water purified by a Nanopure II system (Dubuque, IA).

2.2 Bacteriorhodopsin solubilization and tryptic digestion

Equal aliquots of lyophilized bacteriorhodopsin (100 μg each) were solubilized at 1 μg/μL in five different buffer systems: (a) 50 mM NH_4HCO_3 buffer, (b) 50 mM NH_4HCO_3 buffer containing 60% (v/v) CH_3OH, (c) 50 mM NH_4HCO_3 buffer containing 0.1% PPS, and (d) 50 mM NH_4HCO_3 buffer containing 60% (v/v) CH_3OH and PPS to a final concentration of 0.1% (e) 50 mM NH_4HCO_3 buffer containing 8 M urea. Tryptic digestion was performed at an enzyme-to-protein ratio of 1:20. Solubilization of bacteriorhodopsin using 50 mM NH_4HCO_3 buffer was carried out using sonication in a water bath for 15 min followed by tryptic digestion for 20 h at 37 °C. Solubilization of bacteriorhodopsin using 60% (v/v) CH_3OH was carried as previously described {Blonder, 2004 #66}. Briefly, bacteriorhodopsin was dissolved in 50 mM NH_4HCO_3 buffer containing 60% (v/v) CH_3OH. After sonication in water bath for 15 min solubilized proteins were digested in the same buffer with trypsin at 37 °C for 20 hrs. Solubilization of bacteriorhodopsin using PPS was carried out using manufacturer's recommended procedure. Briefly, lyophilized protein was vortexed in 50 mM NH_4HCO_3 buffer containing 0.1 % PPS followed by sonication in a water bath for 15 min. After incubation at 50 °C the sample was cooled at room temperature and digested with trypsin at 37 °C for 20 hrs. Solubilization of bacteriorhodopsin using 8 M urea was carried out using sonication in water bath for 15 min. Prior to proteolysis the buffer was diluted to 2M urea (final concentration) and digested with trypsin at 37 °C for 20 hrs. Aliquots of 20 μg of bacteriorhodopsin from each of the five digestates were removed at intervals of 0, 0.5, 4, 20 hrs and lyophilized. Solubilization and digestion efficiency of each buffering system was evaluated by SDS-PAGE analysis and visualized using Coomassie blue staining.

2.3 Isolation and Purification of Primary CD14 Human Monocytes

Primary human monocytes were isolated from peripheral blood of healthy donors in accordance with guidelines of the institutional board-approved research protocol. CD14+ monocytes were immuno-affinity purified to ~98% homogeneity as described elsewhere [43]. Briefly, mononuclear cells were separated from blood using standard gradient centrifugation with Ficoll-Hypaque (Pharmacia) followed by CD14+ monocyte purification using immuno-magnetic beads coated with anti CD14 MoAb (Miltenyi Biotech Inc., Auburn, CA) as previously described by Saikh *et al* [43]. Fluorescent antibody cell sorting (FACS) analysis of the purified population demonstrated that ~98% were positive for CD14 expression.

2.4 Monocytes membrane protein isolation, solubilization and proteolysis

Monocytes were lyzed using a combination of hypotonic lysis and sonication while crude membrane fraction was isolated by ultracentrifugation. Briefly, the cell pellet was resuspended in 50 mM NH_4HCO_3 containing 1 mM TCEP and 1 mM PMSF. Cells were homogenized in a Potter-Elvehjem homogenizer followed by ten cycles of 10 second sonication (20 % intensity) using Bronson microprobe sonicator. The homogenate was centrifuged at 5000 × *g* for 5 min to remove unbroken cells and cellular debris. The supernatant was alkylated using 5 mM IAA (final concentration) followed by ultracentrifugation at 100,000 × *g* for 1.5 hrs using a Beckman 50 Ti rotor. Supernatant was discarded and crude membrane fraction subjected to modified carbonate stripping treatment [44]. Briefly, membrane pellet was resuspended in 1 mL of 100 mM Na_2CO_3 (pH 11) and rotated for 2 hrs at 4 °C to eliminate peripheral membrane proteins. After ultracentrifugation at 100,000 × *g* for 1 hr the pellet was washed in d.d.H_2O twice and lyophilized. Three equal aliquots of lyophilized membrane enriched monocyte fraction were resuspended at 1 mg/mL using the three buffer systems: (a) 50 mM NH_4HCO_3 buffer containing 60% (v/v) CH_3OH, (b) 50 mM NH_4HCO_3 buffer containing PPS (c) 50 mM NH_4HCO_3 buffer containing 60% (v/v) CH_3OH and 0.1% PPS. Samples were further solubilized and digested as described

above (section 2.2). After digestion, samples containing PPS were acidified with HCl and incubated at 37 °C for additional one hour to hydrolyze PPS. All samples were lyophilized and dissolved in 0.1% TFA before LC-MS/MS analysis.

2.5 Nano-flow liquid chromatography-tandem mass spectrometry

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed using an Agilent 1100 nanoflow LC system coupled on-line with hybrid linear ion trap-FT-ICR instrument (LTQ-FT, Thermo Electron, San Jose, CA). Reversed-phase columns (75 μm i.d. × 10 cm fused silica capillary with a flame pulled tip) were slurry-packed in-house with 5 μm, 300 Å pore size C-18 stationary phase (Phenomenex, Torrence, CA). After sample injection (2 μg of peptides), the column was washed for 20 min with 98% mobile phase A (0.1% formic acid in water) at a flow rate of 0.5 μL/min. Peptides were eluted from the column using a linear gradient of 2% mobile phase B (0.1% formic acid in ACN) to 60% solvent B in 100 minutes at a flow rate of 0.25 μL/min, then to 98% B for an additional 10 min. The mass spectrometer was operated in a data-dependent mode to automatically switch between MS and MS/MS. The FT-ICR-MS survey scan (*m/z* ranges: 350-1800; 350-750, 740-1000 and 950-1800) was followed by seven MS/MS scans in which the most abundant peptide precursor ions detected in the preceding FT-ICR-MS survey scan were dynamically selected for collision induced dissociation (CID). The threshold of 200 ion counts was used for triggering an MS/MS scan. The normalized CID energy was 35 %; the electrospray voltage was set at 1.6 kV, and the voltage and temperature for the ion source capillary were set at 45 V and 160 °C, respectively.

2.6 Data analysis

All acquired raw data were searched independently against the human protein database (UniProt Human, release 09/2007), using SEQUEST (Thermo, San Jose, CA). The searches were carried out on a Beowulf 18-node parallel virtual machine cluster-computer. Dynamic modifications were added for the detection of the following: carboxyamidomethylated cysteine (+57 Da), and oxidized methionine (+16 Da). For the MS^1 spectra acquired by FTICR-MS the monoisotopic precursor ion mass tolerance was set at 10 ppm Da while for the data dependent MS^2 spectra, acquired by LIT—MS, the fragment ion tolerance was set at 0.5 Da. Only fully tryptic peptides with up to two miscleavages possessing delta correlation $\Delta Cn \geq 0.1$ and charge state dependent cross correlation Xcorr of ≥ 2.0 for $[M+H]^{1+}$, ≥ 2.3 for $[M+2H]^{2+}$ and ≥ 3.75 for $[M+3H]^{3+}$ were considered legitimately identified.

2.7 Transmembrane domain prediction and hydropathicity calculation

Alpha-helical **t**ransmembrane domains (TMD) were mapped using TMHMM [45] available at http://www.cbs.dtu.dk/services/TMHMM while protein grand average of hydropathicity (GRAVY) scores[46] were calculated using ProtParam tool available at the ExPASy Proteomics Server (http://www.expasy.org/tools/protparam.html).

3. Results and discussion

Along with recent advances in MS instrumentation it became evident that existing methods for the isolation, solubilization and proteolysis of membrane proteins are in need of further improvement and optimization [47]. While the mass spectrometer represents a critical component of any proteomic investigation, efficient upstream sample preparation is equally important for successful shotgun analysis of membrane proteins [17]. In addition to efficient isolation of membrane organelles/proteins the solubilization and digestion steps are of critical importance. It is vital to keep extracted membrane proteins solubilized and denatured throughout the proteolysis process [48].

Integral membrane proteins, tied to the membrane bilayer by alpha-helices or attached to it by fatty-acid modifications, require solubilizing conditions different from that of the water-soluble proteins. These characteristics make solubilization, denaturation, and proteolysis an intricate task. The optimization of these conditions for complex membrane protein mixtures is even more challenging as membrane proteins span a wide-range of hydrophobicities and numbers of transmembrane domains (TMDs). Typically, effective solubilization requires the use of solubilizing reagents including detergents, chaotropes or organic solvents to solubilize as large a percentage of the membrane proteome as possible. As an alternative to classic detergents (i.e. SDS, CHAPS), acid-cleavable detergents have recently been proposed for use in membrane proteomics primarily because of their minimal interference with LC separations and/or MS analysis [31,49].

The objective of this study was to find a favorable solubilization and digestion approach for shotgun membrane proteomics of the CD14 human monocyte membrane proteome. We initially compared the efficiency of an *i)* acid-cleavable detergent (PPS), *ii)* an organic solvent system, *iii)* their mixture, and *iv)* a chaotrope (urea) to solubilize and digest a single integral membrane protein (bacteriorhodopsin). Bacteriorhodopsin, a prototypical water insoluble integral membrane protein purified from *Halobacterium halobium* was selected to test the effectiveness of the selected buffer systems. This hydrophobic (GRAVY = 0.723), 262 amino acid protein has seven TMDs and a distinct purple color [50,51]. The 3D structure of bacteriorhodopsin has been resolved at a resolution of 1.55 Å, showing the bulk of protein embedded in the membrane bilayer with short interhelical loops, and short extramembrane N- and C-termini [52]. The purple color shows only when the membrane portion of the protein is intact, (i.e. naturally folded and embedded within the membrane bilayer).

To compare effectiveness, the following buffer systems were employed to solubilize and tryptically digest equal amounts of bacteriorhodopsin: (a) aqueous buffer (50 mM NH_4HCO_3) as a control, (b) organic-aqueous buffer (MeOH in 50 mM NH_4HCO_3), (c) detergent-based buffer (PPS in 50 mM NH_4HCO_3), (d) a combination of both, organic solvent + detergent (MeOH and PPS in 50 mM NH_4HCO_3) and (e) 8 M urea. After solubilization and tryptic digestion, aliquots of 20 μg of digestate were removed from each buffer at 0, 0.5, 4, 20 hrs intervals, analyzed by SDS-PAGE and visualized by Coomassie staining (Figure 1a-e).

On the basis of the staining pattern shown in Figure 1a, most of the membrane protein remained intact when digested in aqueous NH_4HCO_3 buffer. Importantly, we observed that the presence of the purple color of the bacteriorhodopsin solution persisted throughout the 20 h digestion period. This observation suggested that the intramembranous portion of bacteriorhodopsin remained intact since the membrane bilayer was not dissolved, rendering the intramembranous portion of the protein inaccessible to trypsin. In contrast, solubilization and digestion in a mixed organic aqueous buffer (Figure 1b) indicated complete bacteriorhodopsin digestion after 20 h, based on visual analysis. It suggested that dissolution of the membrane bilayer has been successfully achieved along with effective solubilization and denaturation of bacteriorhodopsin, allowing effective tryptic digestion [37]. It is important to note that immediately after CH_3OH (60% v/v) was added to the aqueous bacteriorhodopsin suspension, the purple color faded suggesting the protein had been effectively denatured. The staining pattern depicting solubilization efficiency of the combination of CH_3OH-based buffer and PPS (Figure 1c) indicates complete solubilization and digestion of bacteriorhodopsin after 20 h. The comparison of the density of bacteriorhodopsin bands in figures 1b and 1d suggests a synergistic solubilization/ denaturation effect of organic solvent and PPS, allowing almost complete digestion after 4 hours. This finding is in agreement with the observation reported by Chen et al [35] and

results reported by Bromberg and Klibanov[53]. The staining pattern depicted in Figure 1d shows that significant amount of intact protein remained within the PPS-based buffer even after 20 h of digestion at 37 °C. This suggests that the concentration of PPS used in this investigation is incapable of completely solubilizing or denaturing bacteriorhodopsin. The staining pattern of bacteriorhodopsin solubilized in urea is depicted in Figure 1e. It shows limited bacteriorhodopsin digestion, indicating insufficient solubilization of hydrophobic membrane proteins by urea in this experimental setting [17, 18].

In our second set of experiments we applied *i)* PPS, *ii)* methanol and *iii)* their combination on a membrane proteome isolated from human monocytes to assess the performance of these systems on a complex membrane protein mixture. Since the solubilization and the extent of bacteriorhodopsin proteolysis were poor in urea-based buffer (Figure 1e), this buffer system was not investigated further. Equal aliquots of crude membrane fraction from affinity-purified CD14 human monocytes were solubilized in each selected buffer system. After proteolysis, equal amounts of each digestate (2 μg) were analyzed in triplicate using high-resolution and high-precision LC-MS/MS. It should be pointed out that human monocytes show heterogeneity of protein expression between individuals and between different subpopulations within an individual[54, 55]. However, given the aim of our study, the important issue was to obtain a population of cells that are very highly enriched for what is typically classified as monocytes based on CD14 expression, and we used state-of-the-art technology to obtain an almost completely homogeneous population of monocytes as defined by that criterion. For a study aiming at defining different subpopulations of monocytes based on their membrane protein profiles, additional markers can be used to separate the subpopulations for analysis using our solubilization techniques. In this regard, it is also important to note that our use of primary cells allows us to investigate physiologically relevant conditions. In many cases, the latter cannot be achieved by use of cell lines given the transformations that they have undergone to achieve immortalization. Also, immortalized cell lines typically present a heterogeneous population due to the genomic instability that is common in a number of studied cases.

The number of peptides and proteins identified in the MS analysis using the three compared approaches are shown in Table 1. A total of 1355 peptides identified using the methanol-based approach (Supplementary table 1A) yielded identification of 194 proteins by ≥2 unique peptides (Supplementary Table 1B). A total of 1403 peptides identified using methanol/PPS combination (Supplementary Table 2A) allowed identification of 203 proteins by ≥2 unique peptides (Supplementary Table 2B). PPS alone permitted identification of 1370 tryptic peptides (Supplementary Table 3A) resulting in a total of 216 proteins identified by ≥2 unique tryptic peptides (Supplementary Table 3B). Based on the number of total peptide identifications it is evident that the extent of tryptic digestion is similar across all three approaches. This result is even more evident when the number of unique peptides and proteins identified by at least two peptides is compared (Table 1). Overall, the combination of methanol and PPS yielded the highest peptide/protein ratio of 4.48. The ratio for methanol alone and PPS alone was 3.98 and 3.59, respectively (Table 1). The enrichment of integral membrane proteins was assessed by mapping alpha-helical TMDs for each identified protein within the three datasets using TMHMM software [45].

We first preformed the analysis of the entire human proteome database (UniProt Human, release 09/2007) of 37714 entries which revealed that a total of 8056 (21.36 %) proteins contained at least one or more mapped TMD. The THMM analysis showed that methanol-based approach yielded a total of 141 integral membrane proteins possessing at least one mapped TMD, while the combination of methanol plus PPS and PPS alone yielded 130 and 116 integral membrane proteins, respectively (Table 1). For integral membrane proteins identified by at least two peptides the total number of identified integral membrane proteins

was identical for methanol alone and the methanol/PPS combination (93 proteins each). PPS alone yielded 75 integral membrane protein identifications.

The enrichment of integral membrane proteins estimated by TMHMM software, from protein pools identified by at least two peptides, for methanol alone, methanol/PPS combination, and PPS alone was 47.93 %, 45.81 % and 34.72%, respectively (Table 1). The average number of TMDs per identified integral membrane protein was slightly higher for the methanol/PPS combination (2.8) than for methanol alone (2.7). PPS alone allowed only 2.3 TMDs to be mapped per identified membrane protein suggesting that PPS alone permitted lower protein sequence coverage than the methanol-based approaches. The overlap among the proteins identified using each buffer system is shown in Figure 2.

These results indicate that each approach allowed the identification of different subsets of proteins due to different solubilizing conditions, showing respectable overlap of 67.74 % (63 proteins) between the proteins identified using the two methanol-based approaches. The overlap between the proteins identified using the two PPS-based approaches was lower (i.e. 60%). The enrichment of hydrophobic proteins was assessed by computing the grand average of hydropathicity (GRAVY) index, calculated for each identified protein within the three distinct datasets [46]. The GRAVY index is a global descriptor of protein solubility, and corresponds to the sum of hydrophobicity values for each of the amino-acids in the protein, normalized according to protein length (Note: proteins exhibiting positive GRAVY values were recognized as hydrophobic while proteins exhibiting negative GRAVY values were recognized as hydrophilic)[46]. The number of hydrophobic proteins identified in this investigation by at least two peptides is listed in Table 1. For the methanol-based approach the protein value was 39 (20.1 %), the methanol/PPS combination yielded 43 (21.18%), and 30 (13.88 %) for PPS. Figure 3 shows the GRAVY distributions for all proteins identified using the three compared approaches in relation to overall GRAVY distribution of the human genome. It is evident that methanol alone and methanol/PPS combination showed the ability to enrich for a greater proportion of hydrophobic proteins than PPS alone, which is in agreement with the results shown in Table 1. The correlation of hydrophilic and hydrophobic profiles of the identified proteins to the number of mapped TMDs is shown in Figure 4. Dot diagram analysis showed that methanol alone and the methanol/PPS combination allowed a greater number of integral membrane proteins with higher number of multiple TMDs to be identified than when PPS was used by itself. Overall, the results illustrate significantly higher enrichment of integral membrane proteins when 60% (v/v) methanol-based approaches were employed indicating that this solvent by itself or in combination with PPS represent a potent tool for MS-based membrane proteomics. The average TMD/protein ratio and average GRAVY for of hydrophobic integral membrane proteins suggest a synergistic effect of 60% (v/v) methanol/ 0.1 % PPS combination, which may increase the enrichment of hydrophobic integral membrane proteins. This is in agreement with previous observations [35,49].

To increase the global understanding of membrane function within any cell type, increasing the coverage of membrane proteins identified is an absolute necessity. Blood monocytes play an important role in immuno-regulation and tumor surveillance. However, the molecular mechanisms underlying these biological processes are not well understood [13,56]. A proteomic approach providing significant enrichment of integral membrane proteins should facilitate a better understanding of human monocyte functions in biological systems.

Recently, a global proteomic study of monocytes identified 164 proteins by coupling 2D gel electrophoresis and MS analysis [12]. However, only 12 membrane proteins were identified. By collating data obtained using compared approaches, we have been able to unambiguously

identify a total of 165 unique integral membrane proteins, employing high precision and high resolution MS. Supplementary Table 4 contains broader characterization of these proteins including their function and involvement in human diseases.

Since we were investigating the CD14 human monocyte crude membrane fraction we first sought to determine if the CD14 (monocyte differentiation antigen) was positively identified. CD14 is a hydrophobic (GRAVY = 0.083) surface membrane protein attached to the plasma membrane via a phospholipid anchor [57]. Indeed, CD14 was identified by all of three approaches as shown in Table 2. The two methanol-based approaches allowed unambiguous identification of this protein through multiple peptides, while using PPS alone allowed only a single CD14 peptide to be identified. CD14 is a myelomonocytic differentiation antigen whose gene is located in the “critical” region of chromosome 5. This region is frequently deleted in certain myeloid leukemias [58]. Monocytes are active mediators of inflammation and infection processes [59] and have specificity for lipopolysaccharide (LPS) and other bacteria-wall-derived components. LPS signaling triggers a cascade that leads to cytokine production and shedding of the extracellular domain of CD14.

The buffering of LPS is crucial during acute inflammatory and infectious processes[59]. The number of circulating CD14+ monocytes, and the expression of the CD14 marker by monocytes were found to be significantly lower in patients with septic shock and abnormal hepatocellular function [59,60]. CD14 monocytes in patients with septic shock also exhibited a profound deficiency of TNF-α production, which is considered as hallmark of septic shock[59]. The molecular mechanism of this down-regulation is poorly understood. Thus, further study of CD14 holds a promise for developing a MS-based quantitative assay that might be used to measure CD14 concentration in responses to controlled experimentally evoked stimuli.

The ability to identify peptides residing within the TMD of membrane proteins is an indicator of the extent of a method’s solubilization capability. Microsomal glutathione S-transferase 3 (microsomal GST-3) is 17 kDa, multi-pass hydrophobic (GRAVY = 0.282) integral membrane protein, typically found embedded within endoplasmatic reticulum vesicles. In this study, microsomal GST-3 was identified using both methanol-based approaches but not when PPS was used alone (Table 3). Importantly, two identified peptides (R.IASGLGLAWIVGR.V and R.VLYAYGYYTGEPSKR.S) completely span the 3rd TMD as mapped by TMHMM, of which, R.VLYAYGYYTGEPSKR.S is a highly hydrophobic peptide (GRAVY = 1.308) and resides directly within the TMD. It is suggested that microsomal GST-3 plays significant role in cellular protection against oxidative stress and elimination of xenobiotics [61]. Recently it has been determined by subtractive hybridization screen and validated by northern analysis that after experimentally induced glucose deprivation in human neuroblastoma cells, microsomal GST-3 showed up-regulation by 4 fold [62]. This finding suggests an important role of microsomal GST-3 in the glucopenic response. Evidently, confident identification and significant enrichment of hydrophobic integral membrane proteins using 60% methanol alone or in combination with PPS as shown in Table 4, should allow more detailed characterization of the CD14 monocyte microsomal fraction, if used in the context of multidimensional shotgun analysis [48].

In conclusion, the objective of this investigation was to optimize solubilizing conditions for solution-based shotgun membrane proteomics. We first compared different methods relying on: *i)* 60% buffered methanol *ii)* 0.1% cleavable detergent (PPS), *iii)* 60% buffered methanol/0.1% PPS combination and, *iv)* 8 M urea to solubilize bacteriorhodopsin. Because of inferior performance of urea-based buffer in present experimental setting further

experiments were not performed. Thus, the remaining approaches were then used to solubilize and digest the CD14 human monocyte microsomal fraction. Using high precision MS coupled by nanoflow LC we determined that 60 % buffered methanol alone or in combination with acid-cleavable detergent (PPS) permitted better solubilization/digestion when compared to PPS alone. This was exemplified in significantly higher membrane protein identification/enrichment using both methanol-based approaches. Our results also suggest that the methanol/PPS combination may result in slightly higher enrichment of hydrophobic membrane proteins and might be beneficial for analysis of amount-limited membrane preparations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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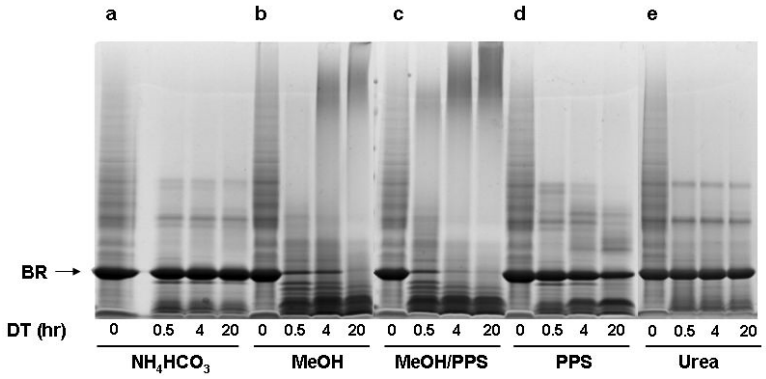

Figure 1.

SDS-PAGE analysis/comparison of solubilization/digestion efficiencies of different approaches, applied on single hydrophobic multipass integral membrane protein bacteriorhodopsin (**br**). Aliquots of 20 μg were removed from each buffer at 0, 0.5, 4, 20 hour intervals during digestion (**dt**) and analyzed on a 4-12% SDS-PAGE followed by Coomassie staining: (**a**) 50 mM NH_4HCO_3 buffer, (**b**) 60% MeOH in 50 mM NH_4HCO_3 (v/v) buffer, (**c**) 0.1 % PPS in 50 mM NH_4HCO_3, (**d**) 60% MeOH/0.1% PPS in 50 mM NH_4HCO_3, (**e**) 8 M urea in 50 mM NH_4HCO_3.

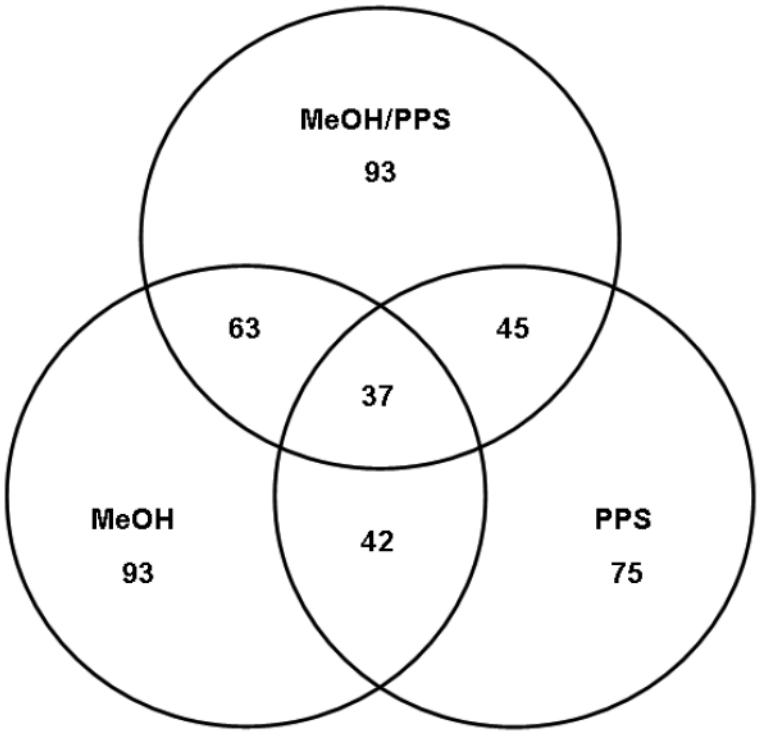

Figure 2.
Triple Venn diagrams illustrating the relationship between the total numbers of integral membrane proteins identified by at least 2 peptides using methanol (MeOH), methanol/PPS combination (MeOH/PPS) and PPS alone.

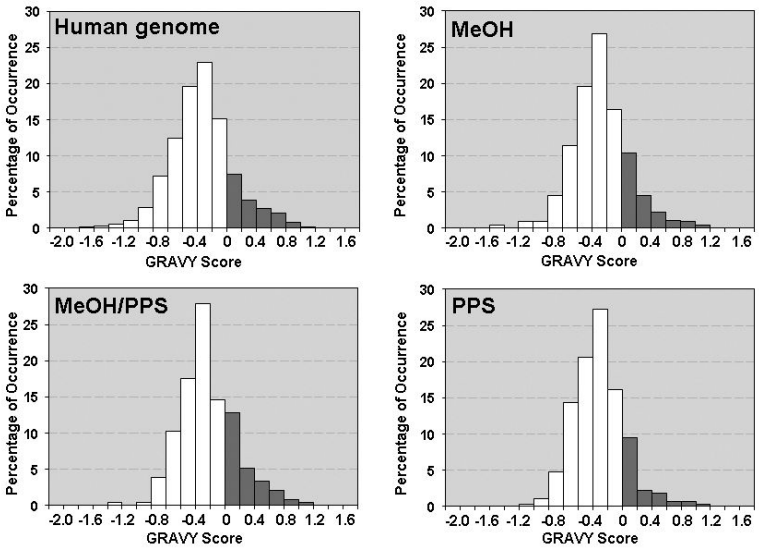

Figure 3.

The relation of hydropathy profiles of the identified proteins using each compared methods and whole human proteome depicted by the GRAVY index analysis. The hydropathy plots for the human proteome, proteins identified using 60 % methanol based method, proteins identified using methanol/PPS combination and proteins identified by PPS only. Each histogram was generated by plotting the number of proteins per 0.2 GRAVY value increment.

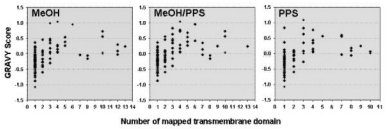

Figure 4.
The relation of hydropathy profiles (GRAVY) and number of predicted TMDs (TMHMM) depicted by dot diagram for identified integral membrane proteins using methanol, methanol/PPS combination and PPS alone.

Table 1

Comprehensive results obtained using three distinctive solubilization approaches: methanol, methanol/PPS combination, and PPS alone

		MeOH	MeOH/PPS	PPS
Peptides: total IDs	a	1355	1403	1370
Peptides: unique IDs	b	680	640	679
Proteins: total IDs	c	340	313	381
Proteins: identified by ≥2 peptides	d	194	203	216
Peptide/protein ratio	a/c	3.98	4.48	3.59
IM* proteins: total IDs	e	141	130	116
Average GRAVY[a]	e	-0.096	-0.102	-0.007
IM proteins ≥2 peptides	f	93	93	75
IM protein enrichment	f/d	47.93%	45.81%	34.72%
Average TMD/protein	f	2.7	2.8	2.3
Hydrophobic IM proteins ≥2 pep	g	39	43	30
Hydrophobic IM protein (%)	g/d	20.10%	21.18%	13.88%
Average GRAVY[a]	g	0.26	0.32	0.24

[a] All identified proteins and the entire human proteome database were analyzed using the ProtParam program (available at http://www.expasy.ch/sprot/sprot-top.html) to calculate the grand average of hydropathicity (GRAVY) for each protein.

Table 2

A list of peptides identifying monocyte differentiation antigen CD14, detected by LC-MS/MS using three distinctive approaches methanol, methanol/PPS combination and PPS alone

MeOH						
Protein name	**MH+** [a]	**Peptide**	**z** [b]	**Max XC** [c]	**Max ΔCN** [d]	**SC** [e]
CD14 precursor	1972.997	R.AFPALTSLDLSDNPGLGER.G	2	2.5298	0.51	1
CD14 precursor	1777.069	R.LTVGAAQVPAQLLVGALR.V	2	3.1222	0.4924	3
CD14 precursor	1933.171	R.RLTVGAAQVPAQLLVGALR.V	2	5.3189	0.7375	2
CD14 precursor	1797.006	R.SWLAELQQWLKPGLK.V	2	3.9901	0.5849	3
MeOH/PPS						
Protein name	**MH+**	**Peptide**	**z**	**Max XC**	**Max ΔCN**	
CD14 precursor	1142.668	K.FPAIQNLALR.N	2	2.7192	0.2607	2
CD14 precursor	1972.997	R.AFPALTSLDLSDNPGLGER.G	2	5.5742	0.5339	3
CD14 precursor	1777.069	R.LTVGAAQVPAQLLVGALR.V	2	3.3594	0.5768	3
CD14 precursor	1797.006	R.SWLAELQQWLKPGLK.V	2	2.8424	0.6037	1
PPS						
Protein name	**MH+**	**Peptide**	**z**	**Max XC**	**Max ΔCN**	
CD14 precursor	2138.189	K.ITGTMPPLPLEATGLALSSLR.L	2	2.5064	0.5238	4

[a] Calculated mass for a given singly-charged peptide.

[b] A charge state for a given peptide.

[c] Maximal Xcorr observed for a given peptide. The cross-correlation score (Xcorr) of the peptide is based on the “fit” of the MS/MS data to the theoretical distribution of ions produced for the peptide.

[d] Maximal ΔCN score observed for a given peptide. The ΔCN score represents a calculated “difference” between the top two Xcorr values for the given peptide.

[e] Total spectral count for a given peptide.

Table 3

Peptides identifying Microsomal GST-3, identified by LC-MS/MS using methanol-based approaches

MeOH						
Gene name	**MH+** [a]	**Peptide**	**z** [b]	**Max XC** [c]	**Max ΔCN** [d]	**SC** [e]
Microsomal GST-3	2974.386	K.YKVEYPIMYSTDPENGHIFNC#IQR.A	3	4.6173	0.6372	1
Microsomal GST-3	1312.774	R.IASGLGLAWIVGR.V	2	3.4382	0.3644	5
Microsomal GST-3	1766.875	R.VLYAYGYYTGEPSKR.S	2	3.3803	0.4787	3
MeOH/PPS						
Gene name	**MH+**	**Peptide**	**z**	**Max XC**	**Max ΔCN**	**SC**
Microsomal GST-3	2974.386	K.YKVEYPIMYSTDPENGHIFNC#IQR.A	3	4.2044	0.6659	1
Microsomal GST-3	1312.774	R.IASGLGLAWIVGR.V	2	3.4553	0.4516	5
Microsomal GST-3	1766.875	R.VLYAYGYYTGEPSKR.S	2	3.4692	0.5654	4

[a] Calculated mass for a given singly-charged peptide.

[b] A charge state for a given peptide.

[c] Maximal Xcorr observed for a given peptide. The cross-correlation score (Xcorr) of the peptide is based on the “fit” of the MS/MS data to the theoretical distribution of ions produced for the peptide.

[d] Maximal ΔCN score observed for a given peptide. The ΔCN score represents a calculated “difference” between the top two Xcorr values for the given peptide.

[e] Total spectral count for a given peptide.

Table 4

A subset of multi-pass hydrophobic integral membrane proteins identified using methanol-based buffers

Acc No	Protein Name	Helices[a]	GRAVY[b]	Cellular location
P12235	ADP/ATP translocase 1	3	0.059	Mitochondrion inner membrane
P00846	ATP synthase a chain	6	0.952	Mitochondrion inner membrane
Q6UW11	ATWD578	3	0.181	Integral to membrane
Q9NX76	CKLF-like MARVEL transmembrane protein	3	0.458	Membrane
Q9BUN8	Derlin-1	5	0.386	Endoplasmic reticulum membrane
Q8TCJ2	Dolichyl-diphosphooligosaccharide--protein	10	0.038	Endoplasmic reticulum membrane
P46977	Dolichyl-diphosphooligosaccharide--protein	13	0.238	Endoplasmic reticulum membrane
Q9BW60	Elongation of very long chain fatty acids protein 1	7	0.352	Endoplasmic reticulum membrane
P33947	ER lumen protein retaining receptor 2	4	0.722	Endoplasmic reticulum membrane
Q9H3K2	Growth hormone-inducible transmembrane protein	6	0.418	Membrane
O75352	Mannose-P-dolichol utilization defect 1 protein	5	0.679	Membrane
O14880	Microsomal glutathione S-transferase 3	4	0.282	Microsome membrane
Q13423	NAD(P) transhydrogenase, mitochondrial precursor	12	0.299	Mitochondrion inner membrane
P61619	Protein transport protein Sec61 subunit alpha isoform 1	10	0.563	Endoplasmic reticulum membrane
O43760	Synaptogyrin-2	4	0.168	Membrane
P30536	Translocator protein	5	0.266	Mitochondrion membrane
Q9UNL2	Translocon-associated protein subunit gamma	4	0.066	Endoplasmic reticulum membrane
Q9BVC6	Transmembrane protein 109 precursor	5	0.541	Nucleus outer membrane
P57088	Transmembrane protein 33	3	0.429	Membrane
Q96CP4	Transporter 1, ATP-binding cassette, sub-family B	7	0.142	Integral to membrane
Q6RW13	Type-1 angiotensin II receptor-associated protein	3	0.482	Endoplasmic reticulum membrane
Q9H1C4	UNC93 homolog B1	12	0.095	Membrane
Q8N357	Uncharacterized protein C2orf18 precursor	10	0.561	Membrane
P27449	Vacuolar ATP synthase 16 kDa proteolipid subunit	4	1.041	Vacuole membrane

[a] A number of transmembrane domains mapped by TMHMM algorithm.

[b] A grand average of hydropathicity (GRAVY) index calculated for a given protein.