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Automated SDS depletion for mass spectrometry of intact membrane proteins though transmembrane electrophoresis

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ABSTRACT: Membrane proteins are underrepresented in proteome analysis platforms because of their hydrophobic character, contributing to decreased solubility. Sodium dodecyl sulfate is a favored denaturant in proteomic workflows, facilitating cell lysis and protein dissolution. However, SDS impedes MS detection and therefore must be removed prior to analysis. Though strategies exist for SDS removal, they provide low recovery, purity, or reproducibility. Here we present a simple automated device, termed transmembrane electrophoresis (TME), incorporating the principles of membrane filtration, but with an applied electric current to ensure near complete (99.9 %) removal of the surfactant, including protein-bound SDS. Intact proteins are recovered in solution phase in high yield (90-100 %), within 1 hour of operation. The strategy is applied to protein standards and proteome mixtures, including an enriched membrane fraction from *E. coli*, resulting in quality MS spectra free of SDS adducts. The TME platform is applicable to both bottom-up MS/MS, as well as LC-ESI-MS analysis of intact proteins. SDS depleted fractions reveal a similar number of protein identifications (285) compared to a non-SDS control (280), being highly correlated in terms of protein spectral counts. This fully automated approach to SDS removal presents a viable tool for proteome sample processing ahead of MS analysis. Data are available via ProteomeXchange, identifier PXD003941.

INTRODUCTION

The analysis of membrane proteins by mass spectrometry is challenged by their hydrophobic character. Compounding with their low levels of expression,[1] membrane proteins have decreased solubility and digestion efficiency relative to their hydrophilic counterparts. Consequently, these important medicinal targets are often underrepresented in proteome analysis platforms.[2–4] Numerous strategies have focused on enhancing the detection of membrane proteins, primarily by addressing their low solubility through inclusion of MS-compatible systems including organic solvents (*e.g.* 60 % methanol),[5] concentrated acid (90 % formic),[6] chaotropes (8M urea),[7] or a multitude of detergents (non-ionic, zwitterionic, volatile, phase transfer, or acid cleavable surfactants).[8–12] Despite these many options, sodium dodecyl sulphate (SDS) remains the preferred choice for membrane protein solubilization[13] and for mass-based proteome separation (*e.g.* SDS PAGE or GELFrEE fractionation).[14] Unfortunately, SDS interferes with downstream analysis of proteins, and is further considered notoriously difficult to deplete.

Even at trace levels, residual SDS will reduce enzyme activity,[15] deteriorate chromatographic resolution,[16,17] and suppress ESI MS signals.[18] LC-MS generally tolerates up to 0.01 % SDS (100 ppm),[18] though some signal deterioration may still be apparent at this level, including formation of SDS adducts in the MS spectrum.[19] To fully enable LC-MS analysis, both free and protein-bound SDS should be eliminated, without sacrificing protein recovery. Dialysis is a classic approach to protein purification, but is insufficient for SDS cleanup ahead of MS; the free energy of binding between SDS and protein (-35 kJ/mol)[20] exceeds that of peptide-hydrogen bonds (~2 to 7 kJ/mol),[21] preventing passive transport of SDS across the dialysis membrane. Other SDS depletion strategies have potential to deplete protein-bound SDS.[22] Among them are column-based methods including HILIC,[23] ion exchange,[24] size exclusion,[25] or affinity approaches.[26] Gel-based electrophoretic strategies retain the protein in a polyacrylamide matrix, facilitating in-gel digestion following detergent removal.[22,27] Protein precipitation is another effective approach to SDS removal.[18,28] We have previously demonstrated high precipitation yields for membrane proteins (> 80%), using cold formic acid to solubilize the protein pellet.[28] Precipitation with CMW varies from 50 to 100 % protein recovery.[29–31] This variation is attributed to accidental loss during pipetting[32] and the partitioning of extremely hydrophobic proteins into the chloroform layer.[33,34] A two-stage filtration and

extraction cartridge has also been reported to facilitate the precipitation process and negates the need for precise pipetting, ensuring high protein purity with reproducible yields.[35] This cartridge format is reminiscent of SDS purification by FASP (filter aided sample preparation),[36] which employs a MWCO filter to retain the larger protein molecules while allowing SDS to spin through. Unfortunately with FASP, upwards of 50% protein loss can be expected,[22,37] owing to nonspecific binding or incomplete digestion.[2] FASP also involves a considerable number of steps, requiring significant time to complete.

In contrast with conventional dialysis, FASP ensures near complete removal of SDS through addition of 8 M urea, in combination with centrifugal force to disrupt SDS-protein binding.[36] FASP would be considered a form of dead-end filtration in which the feedstock is perpendicular to the membrane.[38] In such a strategy, membrane fouling can be an issue, giving rise to reduced analyte recovery.[2,39] Electrofiltration can circumvent this by applying an electric field to oppose the motion of charged particles away from the filter.[40] Cross-flow filtration is another strategy for sample purification wherein the fluid flows tangential to the dialysis membrane.[41] Kim *et al.* recently adopted this technology for SDS depletion in proteomics applications.[19] The purified samples were amenable to MS analysis, though it is noted that minor SDS adducts were still apparent in the MS spectrum.

In this work, we describe a simple electrophoretic device for SDS depletion of membrane proteins. This solution-based process retains proteins behind a MWCO filter, while an applied potential draws the anionic surfactant away from the protein. The approach is distinct from electrofiltration in several ways: first, dead-end filtration is not employed in that there is no significant bulk flow of solution through the membrane; second, the applied electric field directs impurities through the membrane. Operating at constant current, exceptional protein recovery and purity is obtained, with samples being amenable to MS characterization. The approach, which we term transmembrane electrophoresis (TME), offers a fully automated platform for SDS depletion ahead of LC-MS.

EXPERIMENTAL

Instrument Design. An accurate model of the TME device is provided in Figure 1A (the actual device is shown in the abstract image). Referring first to the device core, the sample cell cartridge (**1**) is machined from a 1 cm thick block of Teflon®. Four discrete channels (**2**), each with diameter of 1⁄4", are drilled through the Teflon® plate. Access ports (**3**) are provided from the top of the cartridge and permit transfer of protein solution to the individual cells *via* pipette. Regenerated cellulose dialysis filters (**4**) with nominal MWCO of 3.5 kDa (Fisher Scientific, Ottawa, Canada) are positioned on either side of the sample cartridge, and sealed by custom gaskets (**5**) cast from Sylgard® 184 silicone elastomer (Dow Corning Corp, Midland, MI) to ensure that the cell is water-tight. The cathode (**6**) and anode chambers (**7**) are machined from polyoxymethylene (Delrin®) blocks and accommodate 200 mL of electrolyte (25 mM Tris, 192 mM glycine, pH 8.3). These individual pieces are clamped between two aluminum plates (**8**). Fully assembled, the device measures 7.6 cm high, 10.2 cm wide, by 16 cm long. The system is powered by a PowerPac™ Basic Power Supply (Bio-Rad, Mississauga, Canada), with platinum wires acting as the electrodes.

Sample Preparation. Protein solutions consisting of bovine serum albumin (BSA) or myoglobin (Sigma, Oakville, Canada) were prepared in Milli-Q grade water, purified to 18.2 MΩ·cm, buffered to pH 8.3 with 25 mM Tris, 192 mM glycine (MP Biomedicals, Santa Ana, California), and containing the appropriate concentration of SDS (Bio-Rad). *Escherichia coli* (*E. coli*) proteome extracts were obtained from a fresh cell culture, grown according to established protocols.[42] In brief, *E. coli* was grown in LB media at 37°C with shaking until an OD_{600} of 0.7, and then harvested by centrifugation at 5,000 × *g* (15 min). To isolate the 'membrane enriched' fraction, cells were lysed *via* French Press (2 cycles at 16,000 psi), followed by two rounds of ultracentrifugation as described previously by Wu *et al.*[8] The pellet was suspended in Tris/glycine buffer with 0.5 % SDS, to a final protein concentration of 0.1 g/L by BCA Assay (Pierce, Rockford, IL). The 'whole cell' proteome extract was prepared as follows: *E. coli* cells were snap frozen in liquid nitrogen and lysed through three cycles of sonication with a pellet pestle (30 sec). Proteins were extracted into Tris/glycine buffer and centrifuged (15,000 × *g*, 15 min) to remove cellular debris. The extract was divided into two fractions, one being spiked with SDS to a concentration of 0.5 % (non-SDS fraction as a control). The final protein concentration by BCA was 0.5 g/L.

SDS Removal. To the assembled TME device, 400 µL of SDS-containing protein solution was deposited into each of the four sample cells. The device was run at 40 mA constant current for one hour, with periodic mixing of the sample by pipette throughout the run. SDS-depleted samples were then transferred to an Eppendorf vial. For SDS-depleted membrane enriched protein fractions, the sample cell was subject to an added wash using 300 µL of -20°C formic acid, with brief pipetting to facilitate protein recovery. Residual SDS was quantified through a methylene blue spectroscopic assay,[43] against a calibration curve ranging from 0.5 to 20 ppm SDS. Protein recovery was monitored through a BCA assay using a calibration curve of BSA from 0.25 to 3 µg.

SDS-containing protein solutions were also precipitated in acetone or chloroform/methanol/water (CMW). The acetone protocol is as described previously and used 4 volumes cold acetone,[18] while CMW precipitation is as described by Wessel & Flugge,[44] with minor modifications.[18]

Proteome analysis of *E. coli*. Following SDS depletion, 20 µL portions of the *E. coli* proteome fractions (whole cell or membrane) were combined with 5 µL of Laemmli gel buffer,[45] boiled for 5 min, and loaded into a 12 % T SDS PAGE gel (casting reagents from Bio-Rad), along with control lanes consisting of the equivalent extracts without SDS depletion. Gels were run at 200 V, visualized by silver staining,[46] and imaged with a digital camera. The whole cell fraction was characterized through bottom-up MS. In brief, a portion of the SDS-depleted fraction was solution digested by trypsin (with DTT reduction and alkylation by iodoacetamide) alongside the control samples. The digests were terminated with 10 % TFA, and desalted by reversed phase HPLC on a C18 column.[22] The cleaned peptides were then characterized by LC-MS/MS on a LTQ linear ion trap mass spectrometer (ThermoFisher, San Jose, CA) connected to an Agilent 1200 HPLC system, and employing two replicate injections per sample. The equivalent of 1 µg total protein (assuming 100 % recovery) was loaded onto a 75 µm × 30 cm self-packed C12 column (3 µm Jupiter beads, Phenomenex, Torrance, CA). Peptides were resolved using a 1 hour gradient from solvent A (water / 0.1% formic acid) to solvent B (acetonitrile / 0.1% formic acid) at a flow rate of 0.25 µL/min[47]. The gradient was as follows: 0 min, 5% B; 0.1 min, 7.5% B; 45 min, 20.0% B; 57.5 min, 25% B; 60 min, 35% B; 61 min, 80% B; 64.9 min, 80% B; 65 min, 5% B. The LTQ was operated in data dependent mode. This method cycles from a full MS scan to a zoom scan to determine charge state, followed by MS / MS

of the top three ions, with a collision energy of 35. Charge state screening was enabled to ignore singly charged ions, ions with a charge 4 and greater, or ions where the charge state could not be assigned. The mass range was from 400 – 1300 *m/z*. Dynamic exclusion was applied for 25 s over a range of ± 5ppm.

The membrane enriched *E. coli* protein fractions were analyzed on the LTQ instrument, but as intact proteins (*i.e.* omitting tryptic digestion). Formic acid was removed from the sample by loading the recovered extract on-to a self-packed 1 × 50 mm R2 column (Applied Biosystems), using a temperature programmed gradient described previously by Orton *et al.*,[48] recovering the intact protein as a single fraction. Following partial solvent evaporation, the equivalent of 1 µg total protein was then loaded onto self-packed 100 µm × 100 mm Magic C4 column (300 Å, 5 µm, Michrom Bioresources, Auburn, CA), interfaced to a 75 µm Nanospray Tip (New Objective, Woburn, MA). The LC gradient was as follows: 0 min, 5% B; 5 min, 5%; 6 min, 10%; 25 min, 40%; 35 min, 80%; 36 min, 80%; 37 min, 5%. The LTQ operated in MS-only mode over an *m/z* range 500 to 2000.

TOF-MS Analysis. SDS-depleted myoglobin standards were analyzed on a Bruker MicroTOF system (Billerica, MA). A 5 µL portion of the sample was injected onto a 1 × 100 mm Magic C4 column, with temperature held constant at 75°C using an Agilent 1100 HPLC system. The mobile phase delivered an isocratic flow of 50 % ACN, 0.1 % formic acid in water at 150 µL/min, with a 1:10 post-column split allowing 15 µL/min to be directed to the ESI source of the instrument. The ESI source operated in positive mode with capillary voltage of 4 kV, nebulizer gas 1 bar, dry gas 5 L/min, and drying temperature of 180°C. The transfer exit capillary was 150 V, transfer time 80.0 µs, and Hexapole RF 800 Vpp.

Data analysis. MS/MS spectra of the *E. coli* proteome fractions (whole cell and membrane enriched) were searched by Proteome Discoverer software against the SEQUEST *E. coli* database (downloaded May 2014, 4269 entries), with modifications of oxidized methionine, carbamidomethylation at cysteine, and up to 2 missed cleavages. The mass tolerance was 1 Da (MS mode) and 0.8 Da (MS/MS mode), assigning a peptide false positive rate of 1%. Proteins were further screened by requiring a minimum 2 unique peptides from a given sample. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[49] partner repository with the dataset identifier PXD003941 and 10.6019/PXD003941. Cellular compo-

nents were determined using Gene Ontology functional annotation from DAVID (Database for Annotation, Visualization, and Integrated Discovery).[50,51] Hydrophobicity was determined from GRAVY scores[52] obtained from www.bioinformatics.org/sm2/protein_gravy.html, while transmembrane topology was predicted using web-based software employing the TMHMM[53] and AmphipaSeeK[54] algorithms. ImageJ was used to quantify recovery of *E. coli* membrane proteins from the SDS-PAGE image. The ESI-MS spectra of intact proteins were deconvoluted with software written in MS Excel.

Safety Considerations. The TME apparatus is a high voltage instrument, operating without approved safety interlock. Extreme caution should be taken to avoid accidental shock. Prior to handling solutions in the device, the unit is unplugged from the power supply.

RESULTS AND DISCUSSION

SDS depletion. The principles of transmembrane electrophoresis are visualized in Figure 1B. SDS-containing protein solution are inserted into a sample cell, bordered at either side by a MWCO membrane. A uniform Tris/glycine buffer system (pH 8.3) is applied to the cathode and anode chambers. Upon application of current, the anionic detergent migrates towards the anode. At this pH, most proteins also adopt a negative charge, though the porosity of the membrane (3.5 kDa) confines these larger molecules to the sample cell. As is shown below, the device is capable of depleting not only free surfactant monomers but also removes protein-bound SDS.

A series of time course experiments were conducted, monitoring SDS depletion over a one-hour period. The current ranged from 0 and 50 mA. As shown in Figure 2, higher currents increasing the rate of SDS depletion from the 0.5 g/L BSA solution. In the absence of current (0 mA) a minor reduction of SDS is observed, dropping from 0.5 % to a final concentration of 0.3 % (3,000 ppm). Being representative of conventional dialysis, protein-bound SDS is not expected to be removed. With a total protein load of 150 µg in 300 µL, the equilibrium binding of SDS to protein translates to 700 ppm detergent. As seen in Figure 2, 1 hour at 20 mA drives the residual

SDS well below 700 ppm, indicating both free and protein-bound SDS are being removed from the sample solution. This clearly distinguishes transmembrane electrophoresis from conventional dialysis. At 30 mA, residual SDS falls below the 100 ppm critical threshold.[18] Following one-hour operation at 40 mA, the final SDS concentration was quantified as 4.7 ± 3 ppm, while 50 mA depleted SDS to 0.8 ± 1 ppm. The limit of quantitation of the methylene blue assay is 2.5 ppm (LOD 0.5 ppm), which contributes to the errors shown in the figure. As shown in these constant current experiments, it is clear that TME is capable of near complete removal of SDS in a reproducible fashion.

Protein recovery. So long as the current did not exceed 40 mA, SDS depletion occurs with minimal loss of analyte. BSA recovery was quantified above 90% in all cases (see supplemental Figure S1A). However, as current increased to 50 mA, protein recovery dropped to 60 %. These results are explained by considering the temperature of the solution in the sample cell. At 40 mA, the sample temperature rose from 16 to 34ºC over the 1 hour experiment. At 50 mA the temperature rose to 60°C (see supplemental Figure S1B). A high temperature increase is indicative of the high solution resistance as ions traverse the MWCO membranes, together with a low degree of heat dissipation. Using concentrated BSA solutions, protein deposits are visibly apparent on the MWCO membrane at 50 mA. Membrane fouling is a well-known phenomenon observed during electrofiltation.[41] However, to avoid this occurrence and ensure high protein recovery together with a high degree of purity, the TME device is operated at 40 mA constant current for one hour.

Less protein and more SDS. The optimized protocol for SDS depletion was applied to samples containing higher initial SDS concentrations (up to 2 %), and at 10-fold lower protein concentration (0.05 g/L, or 15 µg BSA). Figure 3 summarizes the results. Despite higher initial SDS, TME successfully removes the detergent to below 10 ppm (Figure 3A). This level of depletion compares favorably to other protocols for SDS depletion.[22] The protein recovery values are shown in Figure 3B. At lower protein concentration, recovery was statistically indistinguishable from 100 % regardless of the initial concentration of SDS. Interestingly, recovery was superior at lower protein concentration, and recovery remained above 90 % for all trials. This again compares favorably over alternative methods of SDS depletion.[22]

Mass spectrometry of SDS-depleted proteins: Myoglobin was employed as a test sample and subject to LC-MS analysis as the intact protein. As seen in Figure 4, though a charge envelope can still be obtained, a control solution containing 100 ppm SDS (Fig 4B) shows considerable signal degradation compared to the 10 ppm spiked control (Fig 4A). The maximal tolerance of LC-MS towards SDS is not an absolute value and depends on the amount of protein analyzed, together with the instrumental operating conditions. Acetone precipitation readily depletes SDS below 100 ppm.[18] As shown in Figure 4C, though the intensity of the myoglobin charge envelope is restored, SDS adducts are now clearly visible in the MS spectrum. By contrast, following SDS depletion by TME, no SDS adducts are visible in the MS spectrum of myoglobin (Figure 4D). Based on these results, transmembrane electrophoresis is capable of removing SDS from protein samples to levels favorable for LC-MS analysis.

Application to proteome analysis. We employed an *E.coli* 'whole cell' proteome extract spiked with 0.5% SDS as a representative mixture, comparing the TME purified sample to an equivalent extract prepared in the absence of SDS. The gel image displayed in Figure 5 demonstrates the high recovery observed over a wide range of molecular weights (10 - 200 kDa) following TME purification. A detailed list of proteins identified by bottom-up MS, together with peptide spectral counts from replicate analysis of independently purified fractions is found in supplemental Table S1. Examining the Venn diagram in Figure 5, the majority of proteins identified (79 %) were common to both the SDS-depleted and control samples. A more in-depth comparison of the proteins recovered from the SDS-depleted samples is afforded by plotting the peptide spectral counts of discrete proteins relative to the control sample. The graph in Figure 5 plots the average protein spectral counts in the control and SDS-depleted samples. The numbers of spectral counts per protein are highly correlated between the control and SDS-depleted samples (R^2= 0.93). With the SDS-depleted samples as the ordinate, we note the slope of the linear regression line is above 1 (slope = 1.08 to 1.12 at 95 % confidence). This indicates a preference towards detecting a greater number of peptides in the SDS-depleted sample. This result can be explained in a number of ways. First, the recovery of protein in our optimized SDS-depletion experiments is expected to be high, as confirmed in part from the SDS PAGE gel image. Second, we have previously reported that trace levels of SDS (~10 ppm) contribute a minor enhancement to MS signals for electrosprayed peptides.[17] It is also possible that dena-

turation of the protein samples, contributed by the initial presence of SDS, could enhance digestion efficiency. These results clearly demonstrate the utility of transmembrane electrophoresis as a front-end technology for SDS depletion ahead of bottom-up MS analysis of complex proteome mixtures.

Application to membrane proteins: Unlike water-soluble proteins, the depletion of SDS from a mixture of membrane proteins increases the risk of sample loss, as these proteins may not remain soluble in the absence of detergent. Fortunately, given the design of the transmembrane electrophoresis device, all proteins will remain confined to the sample cell, including those that may precipitate once SDS is removed. Such proteins would tend to aggregate on the dialysis membrane of the TME device, though this does not imply that this aggregation is irreversible. As demonstrated below, inclusion of an appropriate solvent to wash the sample cell is sufficient to recover such proteins. Here, we include a rapid wash of the sample cell with cold (-20°C) formic acid. Our group has previously shown the effectiveness of this solvent to rapidly resolubilize precipitated membrane proteins, being as effective a solvent as employing 1 % SDS with extended sonication.[28] Maintaining a reduced temperature prevents protein formylation, which otherwise occurs when samples are exposed to formic acid.[28]

Figure 6 illustrates the protein recovery obtained following SDS depletion of an enriched *E. coli* membrane proteome extract. The gel lanes labelled 'water' represents those proteins directly recovered from the solution phase of the sample cell following SDS depletion (final SDS concentration 2.1 ± 0.3 ppm). Unlike the *E. coli* 'whole cell' fraction described above, protein recovery from the membrane-enriched fraction was considerably reduced (< 25 % based on the band intensity relative to the control lanes). Recovery was also largely variable between sample cells (gel lanes i and ii of Figure 6), depending on the degree of protein aggregation that occurs in the absence of SDS. However, as shown from Figure 6, inclusion of a formic acid wash recovers a significantly greater percentage of the sample. Combined with the water fraction, the band intensity of the gel accounts for 87 ± 7 % of the proteins recovered following SDS depletion. We note that some gel bands are of higher intensity in the water fractions (*e.g.* the dark band near ~ 27 kDa). Water-soluble (cytosolic) proteins may be present in

the membrane-enriched fraction. Alternatively, certain membrane or membrane-associated proteins may still remain in solution in the absence of SDS.

The composition of proteins recovered from the membrane-enriched protein fraction was assessed through bottom-up MS/MS. The water and formic fractions obtained from TME purification were analyzed independently, and the resulting lists of identified proteins are provided in supplemental Table S2. In total, 218 unique proteins were identified from these fractions. By comparison using an identical MS platform from our lab, an equivalent *E. coli* membrane preparation has previously yielded 192 proteins following acetone precipitation or 137 total proteins with CMW precipitation to deplete SDS.[28] Analysis of the identified proteins demonstrates the proteins recovered through TME-purification are indeed enriched in membrane proteins. Gene Ontology mapping confirms that 59 % of the 218 identified proteins are described as membrane or membrane-associated (Figure 7A). The enrichment of membrane proteins agrees with previous data from our lab wherein 17 % of identified proteins from the *E. coli* whole cell fraction are membrane or membrane associated, while 53 % are detected in the membrane enriched fraction.[28] Inspection of the list of identified proteins further reveals membrane proteins to be among the most abundant in the sample, attributing the highest number of peptide spectral counts (PSM). Outer membrane proteins A (ompA) and C (ompC) were identified with the highest PSM in both the water and formic fractions. These transmembrane β barrel porins are highly expressed in *E. coli* and are therefore expected to be among the proteins identified in the membrane enriched fraction. The 128 proteins characterized as membrane or membrane-associated, together with those having an uncharacterized designation (50 more), were further assessed using TMHMM[53] and AmphipaSeek[54] algorithms. As shown in Figure 7B, 102 (57 %) contained in-plane membrane (IPM) anchoring points and 70 (39 %) contained predicted transmembrane segments. Proteins with transmembrane segments are generally more hydrophobic than their counterparts.[2] As an example, among the identified proteins, we observed guanine/hypoxanthine permease (GhxP), an inner membrane transport protein which was correctly predicted by TMHMM of possessing 12 alpha helical transmembrane segments. This protein has a GRAVY score above +1 (see Table S2), and so was expected to be poorly soluble in an SDS-free buffer. Interestingly, this protein was observed in both the water and formic fractions. While one might expect to recover a greater portion of such hydrophobic proteins in the formic acid

fraction, our data does not support this hypothesis. And while there were no apparent differences in trends for the molecular weight, isoelectric point, or hydrophobicity across the two fractions (supplemental Table S2), this result is easily explained by noting that the vast majority of proteins observed in the water fraction (109 of the 117 identifications) were also detected in the formic fraction. The proteins recovered in the water fraction following SDS depletion may not necessarily be dissolved in solution, as aggregates may still be dispersed in the sample. Nonetheless, nearly twice as many proteins were identified in the formic fraction compared to the water fraction (210 *vs.* 117). From these results, with no specific bias towards the type of protein recovered in the two fractions, the water and formic acid wash could easily be combined into a single sample for subsequent MS analysis. It is also concluded that hydrophobic membrane proteins are amenable to bottom up MS analysis following TME purification to deplete the sample of SDS.

Mass Spectrometry of Intact Proteins. One of the significant advantages of TME is its amenability to top-down workflows. Here we demonstrate LC-MS analysis of intact proteins recovered from these same *E. coli* membrane enriched protein fractions following TME purification. Figure 8A shows the TIC trace and a selection of charge state envelopes (insets) for intact proteins recovered from the formic acid wash (TIC of the water fraction shown in supplemental Figure S2). As a basis for comparison, the equivalent sample was depleted of SDS through CMW precipitation, a reliable and effective approach previously demonstrated to recover intact membrane proteins in high yield.[18] This TIC trace is shown in Figure 8B. Together with the water fraction, all TIC traces show similar chromatographic features, including a dominant peak at ~ 40 min. Deconvolution of the MS data at this retention time provided a molecular weight of 35,165 ± 5 Da in the formic fraction, which agrees with the mass of outer membrane protein A (35,166 Da), which we also observed in high relative abundance through bottom up MS analysis. Deconvolution of the charge envelopes reveals several common proteins detected across the three fractions, though some masses were uniquely detected (supplemental Table S3). Regardless of the sample purification approach, no SDS adducts were observed. Though formic acid is known to covalently modify proteins,[55,56] the use of cold formic acid preserved the unmodified mass of the protein,[28] as formylation events (+28 Da) were also not detected (supplemental Figure S3). The distinct charge state enve-

lopes observed with high signal to noise for multiple proteins demonstrates the ability to incorporate TME into an intact protein workflow.

CONCLUSION

The results presented here clearly demonstrate the utility of transmembrane electrophoresis (TME) as a front-end technology for SDS depletion ahead of MS analysis. Transmembrane electrophoresis is a simple and effective technology for SDS depletion, requiring no user manipulation beyond loading the sample into and out of the device. The design provides exceptional protein recovery as even precipitate proteins are readily recovered from the sample chamber with a simple washing step. Transmembrane electrophoresis provides a level of protein recovery and purity that exceeds that of alternative purification strategies (including protein precipitation). SDS is consistently depleted to levels permitting MS analysis following tryptic digestion, or direct analysis of intact proteins. Though true top-down proteome analysis entails tandem MS, the generation of intense charge state envelopes, free of SDS adducts, indicates the potential for this device in such a workflow. Device automation ensures consistent and timely processing of multiple samples (currently 4 at a time). Preliminary observations of TME suggest that other parameters (sample additives, buffer composition, dimensions of the sample cell, *etc.*) influence the rate of SDS depletion, as well as recovery of proteins, sample output, and operation time. These and other parameters of transmembrane electrophoresis will be reported in the future.

FIGURES

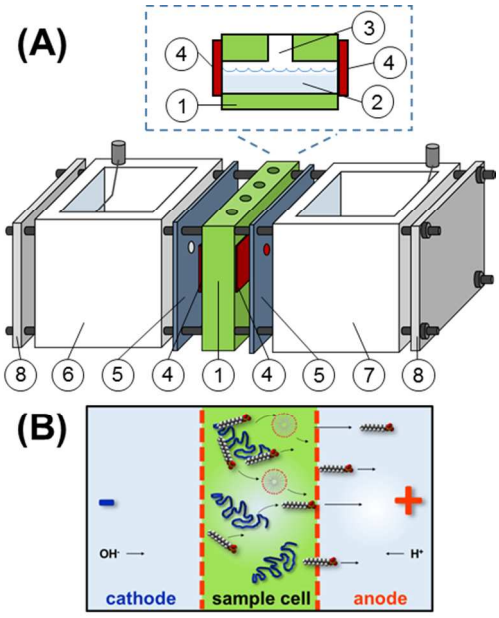

Figure 1. (A) An accurate model of the SDS depletion apparatus. The sample cell cartridge (1) comprises four discrete channels (2) bordered by MWCO filters (4). Additional descriptions of the device are provided in the experimental. **(B)** Anionic SDS is driven across the membrane towards the anode by an applied electric field. Both free and protein-bound SDS monomers are depleted, while intact proteins are confined to the sample cell.

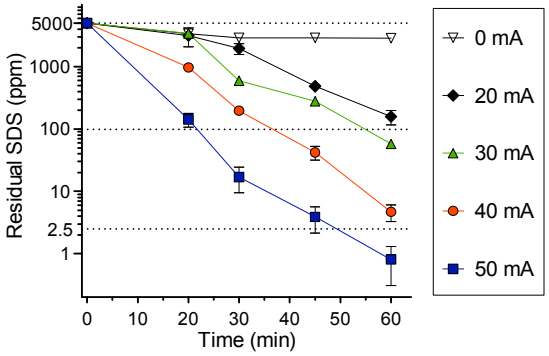

Figure 2. Time course of SDS depletion as a function of the applied current. Solutions initially comprised 150 µg BSA in 0.5 % SDS (5000 ppm). The critical value that permits LC-MS/MS analysis is indicated (100 ppm), as is the limit of quantitation of the methylene blue assay (2.5 ppm) used to monitor residual SDS. Error bars represent standard error of four replicates.

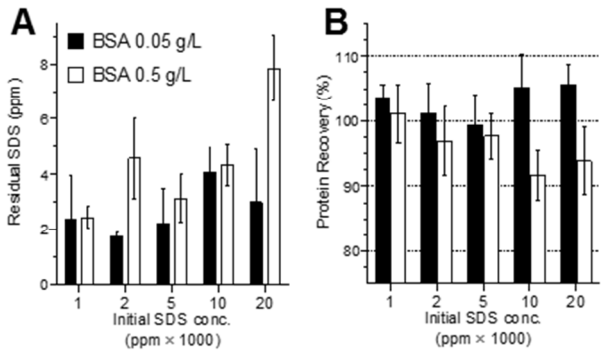

Figure 3. (**A**) Residual SDS and (**B**) protein recovery observed following SDS depletion (1 hour, 40 mA), as a function of the initial surfactant and protein concentration. Error bars represent standard error for depletion of four independent samples.

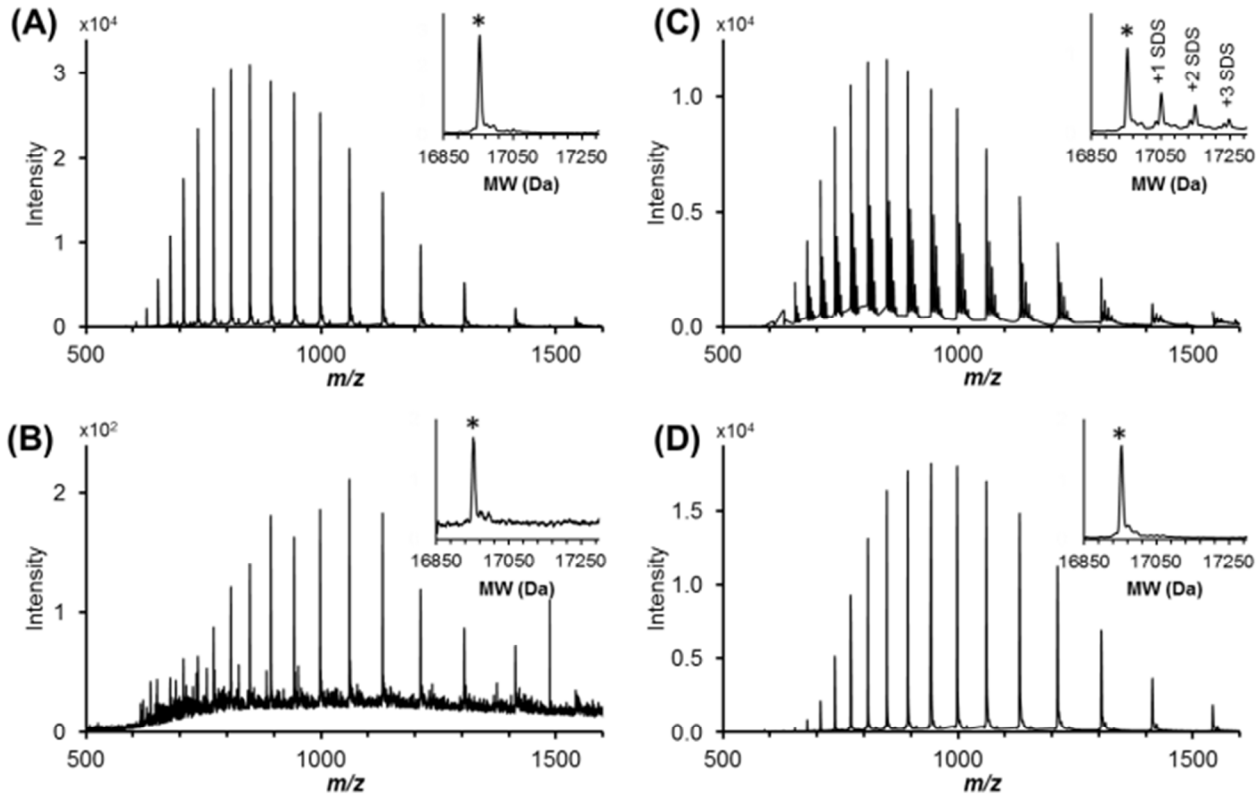

Figure 4. MS spectra of myoglobin, spiked with **(A)** 10 ppm SDS or **(B)** 100 ppm SDS. **(C)** SDS is depleted from the protein via acetone precipitation (initial 0.5% SDS). **(D)** SDS is depleted from an equivalent sample via trans-

membrane electrophoresis. Insets show the deconvoluted spectra, with the labelled peak (*) corresponding to the unmodified protein (16,951 Da).

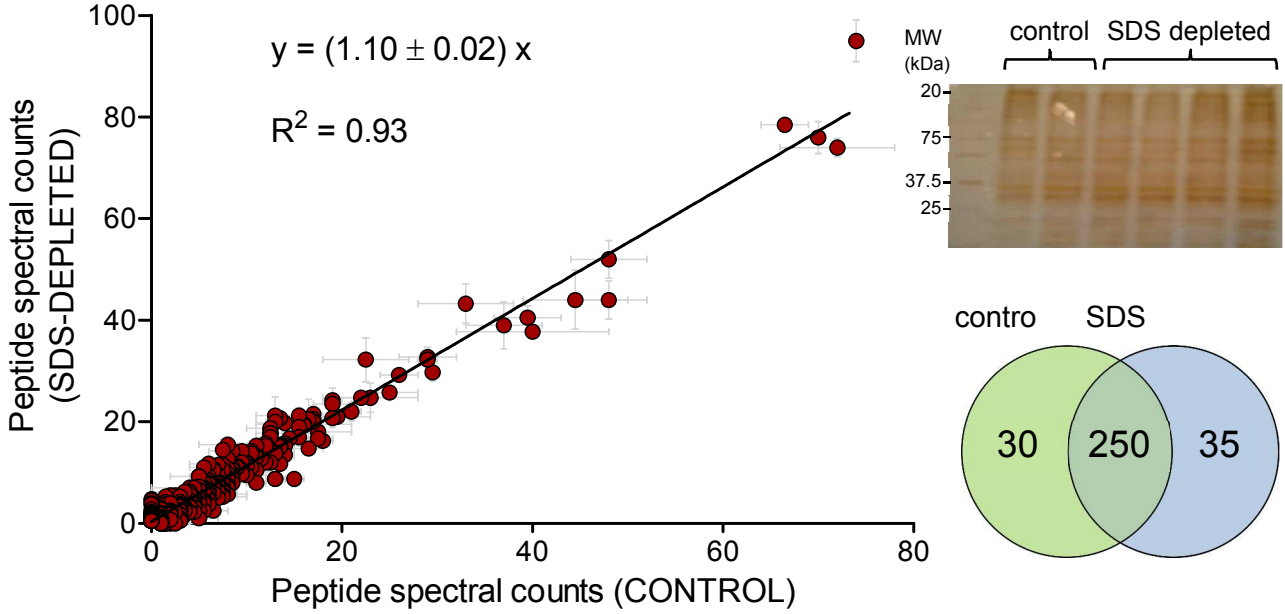

Figure 5. Comparison of proteomic data for SDS-depleted *E. coli* extract in 0.5 % SDS relative to a control, prepared in the absence of SDS. SDS PAGE (top right) shows the band intensity of the depleted fractions to be similar to the control. The Venn diagram (bottom right) summarizes the number of proteins identified by LC-MS/MS from the control *vs* SDS depleted fractions. The graph at left plots the average number of peptide spectral counts observed from replicate analysis of the control (N=2) compared to the SDS-depleted fractions (N=4). A slope above 1 indicates enhanced peptide detection in the SDS-depleted fractions. Error bars represent the standard error for replicate MS analysis of equivalent fractions.

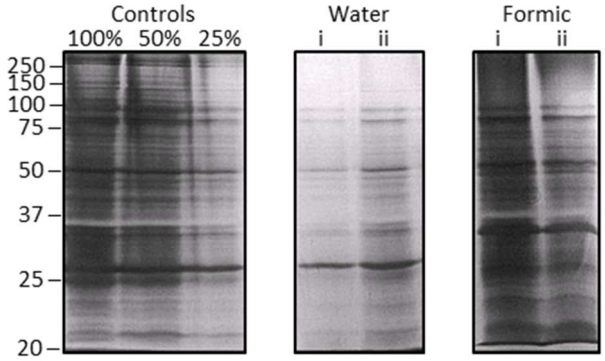

Figure 6. The *E. coli* membrane proteome extract as visualized by SDS-PAGE.[46] Controls are for the equivalent sample without TME purification and depict a theoretical recovery of 100, 50, or 25 %. Proteins directly recovered in the 'water' fraction from independent sample replicates (i or ii) with TME are shown. The 'formic' shows the additional proteins that were recovered from the same sample cells (i or ii), following a wash with 80 % cold formic acid.

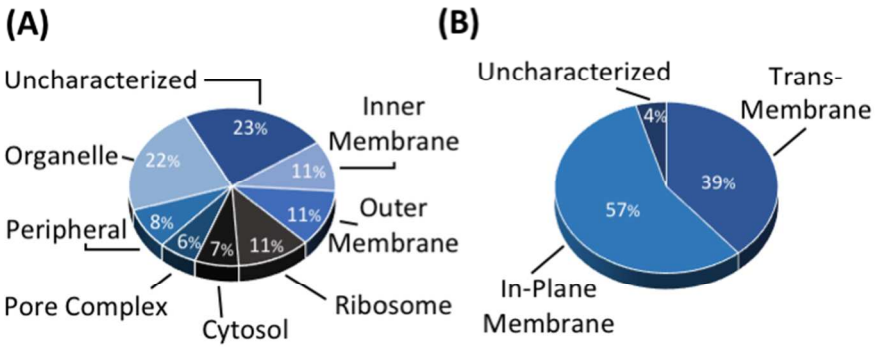

FIGURE 7. **(A)** The cellular compartments of the recovered proteins as profiled by the Gene Ontology function in DAVID. **(B)** All proteins, except those identified as ribosomal or cytosolic, were further assessed based on their interactions with the membrane and characterized as containing 1 to 16 transmembrane segments with TMHMM,[53] or 3 to 42 in-plane membrane (IPM) anchors identified with AmphipaSeek.[54]

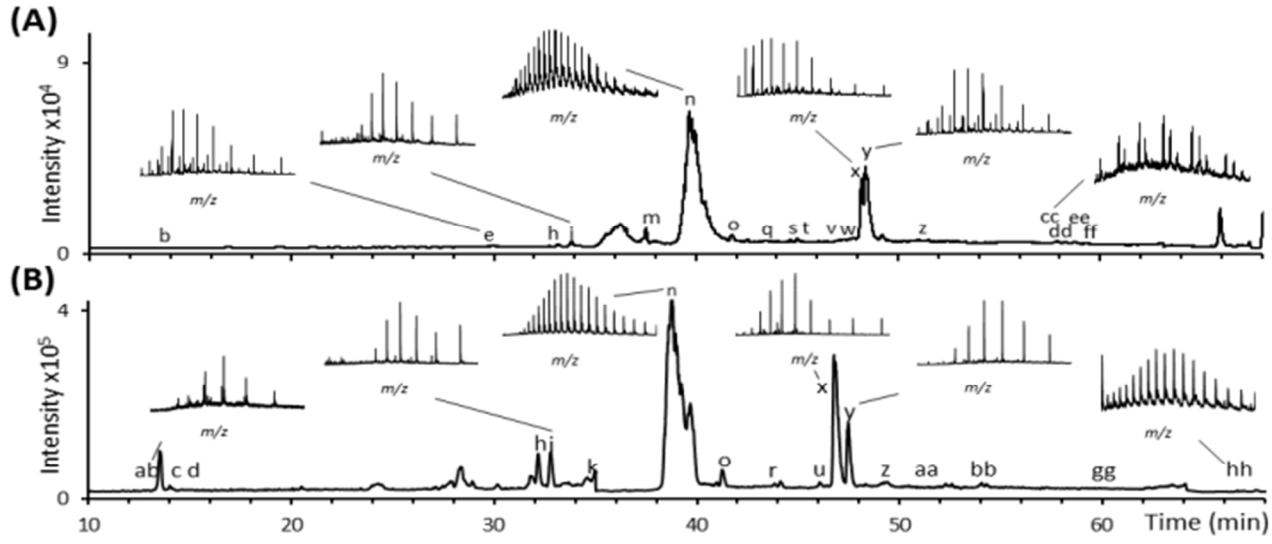

Figure 8. TIC traces for *E. coli* membrane extracts from analysis of **(A)** the formic acid fraction recovered from TME, and **(B)** CMW precipitation. Letters correspond to distinct charge state envelopes. A selection of spectra are shown. A complete list of observed proteins is provided in supplemental Table S3.

ASSOCIATED CONTENT

Supporting Information. The following files are available free of charge via the Internet at http://pubs.acs.org.

Figure S1: (A) Protein recovery following 1 hour SDS depletion at varying currents. Samples initially comprised 0.5 g/L BSA (300 µL). Error bars represent standard error of four independent samples. (B) The temperature of the sample cell was monitored over the course of the SDS depletion experiments. (PDF)

Figure S2: TIC traces for *E. coli* membrane extracts from analysis of the water fraction recovered from TME. Letters correspond to distinct charge state envelopes. A selection of spectra are shown (deconvoluted as insets). (PDF)

Figure S3: Deconvoluted MS spectra for select proteins recovered in the formic acid fraction following SDS depletion by TME. No SDS adducts are visible, indicating that the detergent was successfully depleted. The absence of an observable +28 Da peak demonstrates how cold formic acid can be used to solubilize proteins while preserving the native structure. (PDF)

Table S1: Proteins identified from *E. coli* 'whole cell' fraction by bottom-up MS, together with peptide spectral counts (TME purified *vs.* control). (XLS)

Table S2: Proteins identified from *E. coli* 'membrane' fraction by bottom-up MS, recovered from the water and formic acid fractions following SDS depletion. (XLS)

Table S3: Deconvoluted molecular weight of proteins observed by LC-MS from *E. coli* membrane fraction following TME purification as recovered in the water and formic acid fractions, or following CMW precipitation to deplete SDS. (XLS)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest

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REFERENCES

(1) Wiśniewski, J. R.; Rakus, D. Multi-enzyme digestion FASP and the "Total Protein Approach"-based absolute quantification of the Escherichia coli proteome. *J. Proteomics* **2014**, *109*, 322–331.

(2) Giannone, R. J.; Wurch, L. L.; Podar, M.; Hettich, R. L. Rescuing those left behind: Recovering and characterizing underdigested membrane and hydrophobic proteins to enhance proteome measurement depth. *Anal. Chem.* **2015**, *87*, 7720–7728.

(3) Speers, A. E.; Wu, C. C. Proteomics of integral membrane proteins--theory and application. *Chem. Rev.* **2007**, *107*, 3687–3714.

(4) Vuckovic, D.; Dagley, L. F.; Purcell, A. W.; Emili, A. Membrane proteomics by high performance liquid chromatography-tandem mass spectrometry: Analytical approaches and challenges. *Proteomics* **2013**, *13* (3-4), 404–423.

(5) Blonder, J.; Conrads, T. P.; Yu, L. R.; Terunuma, A.; Janini, G. M.; Issaq, H. J.; Vogel, J. C.; Veenstra, T. D. A detergent- and cyanogen bromide-free method for integral membrane proteomics: application to Halobacterium purple membranes and the human epidermal membrane proteome. *Proteomics* **2004**, *4* (1), 31–45.

(6) Schindler, P. A.; Van Dorsselaer, A.; Falick, A. M. Analysis of hydrophobic proteins and peptides by electrospray ionization mass spectrometry. *Anal. Biochem.* **1993**, *213*, 256–263.

(7) Gordon, J. A.; Jencks, W. P. The relationship of structure to the effectiveness of denaturing agents for

proteins. *Biochemistry* **1963**, *2* (1), 47–57.

(8) Wu, F.; Sun, D.; Wang, N.; Gong, Y.; Li, L. Comparison of surfactant-assisted shotgun methods using acid-labile surfactants and sodium dodecyl sulfate for membrane proteome analysis. *Anal. Chim. Acta* **2011**, *698* (1-2), 36–43.

(9) Kadiyala, C. S. R.; Tomechko, S. E.; Miyagi, M. Perfluorooctanoic acid for shotgun proteomics. *PLoS One* **2010**, *5* (12), e15332.

(10) Masuda, T.; Tomita, M.; Ishihama, Y. Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis. *J. Proteome Res.* **2008**, *7* (2), 731–740.

(11) Garavito, R. M.; Ferguson-Miller, S. Detergents as tools in membrane biochemistry. *J. Biol. Chem.* **2001**, *276* (35), 32403–32406.

(12) Le Maire, M.; Champeil, P.; Møller, J. V. Interaction of membrane proteins and lipids with solubilizing detergents. *Biochim. Biophys. Acta* **2000**, *1508*, 86–111.

(13) Bereman, M. S.; Egertson, J. D.; Maccoss, M. J. Comparison between procedures using SDS for shotgun proteomic analyses of complex samples. *Proteomics* **2011**, *11* (14), 2931–2935.

(14) Tran, J. C.; Doucette, A. A. Gel-eluted liquid fraction entrapment electrophoresis: An electrophoretic method for broad molecular weight range proteome separation. *Anal. Chem.* **2008**, *80* (5), 1568–1573.

(15) Arribas, J.; Castano, J. G. Kinetic studies of the differential effect of detergents on the peptidase activities of the multicatalytic proteinase from rat liver. *J. Biol. Chem.* **1990**, *265* (23), 13969–13973.

(16) Kawasaki, H.; Suzuki, K. Separation of peptides dissolved in a sodium dodecyl sulfate solution by reversed-phase liquid chromatography: Removal of sodium dodecyl sulfate from peptides using an Ion-exchange precolumn. *Anal. Biochem.* **1990**, *186* (2), 264–268.

(17) Vieira, D. B.; Crowell, A. M. J.; Doucette, A. A. Perfluorooctanoic acid and ammonium perfluorooctanoate: Volatile surfactants for proteome analysis? *Rapid Commun. Mass Spectrom.* **2012**, *26* (5), 523–531.

(18) Botelho, D.; Wall, M. J.; Vieira, D. B.; Fitzsimmons, S.; Liu, F.; Doucette, A. Top-down and bottom-up proteomics of SDS-containing solutions following mass-based separation research articles. *J. Proteome Res.* **2010**, *9*, 2863–2870.

(19) Kim, K. H.; Compton, P. D.; Tran, J. C.; Kelleher, N. L. An on-line matrix removal platform for coupling gel-based separations to whole protein electrospray ionization mass spectrometry. *J. Proteome Res.* **2015**, *14* (5), 2199–2206.

(20) Chattoraj, D. K.; Biswas, S. C.; Mahapatra, P. K.; Chatterjee, S. Standard free energies of binding of solute to proteins in aqueous medium. Part 2. Analysis of data obtained from equilibrium dialysis and isopiestic experiments. *Biophys Chem* **1999**, *77* (1), 9–25.

(21) Sheu, S.-Y.; Yang, D.-Y.; Selzle, H. L.; Schlag, E. W. Energetics of hydrogen bonds in peptides. *Proc. Natl. Acad. Sci.* **2003**, *100* (22), 12683–12687.

(22) Kachuk, C.; Stephen, K.; Doucette, A. Comparison of sodium dodecyl sulfate depletion techniques for

proteome analysis by mass spectrometry. *J. Chromatogr. A* **2015**, *1418*, 158–166.

(23) Alpert, A. J. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J. Chromatogr.* **1990**, *19* (499), 177–196.

(24) Weber, K.; Kuter, D. J. Reversible Denaturation of Enzymes by Sodium Dodecyl Sulfate. *J. Biol. Chem.* **1971**, *246* (14), 4504–4509.

(25) Amons, R.; Schrier, P. Removal of sodium dodecyl sulfate from proteins and peptides by gel filtration. *Anal. Biochem.* **1981**, *116* (2), 439–443.

(26) Hengel, S. M.; Floyd, E.; Baker, E. S.; Zhao, R.; Wu, S.; Paša-Tolić, L. Evaluation of SDS depletion using an affinity spin column and IMS-MS detection. *Proteomics* **2012**, *12* (21), 3138–3142.

(27) Liu, Y.; Lin, Y.; Yan, Y.; Li, J.; He, Q.; Chen, P.; Wang, X.; Liang, S. Electrophoretically driven SDS removal and protein fractionation in the shotgun analysis of membrane proteomes. *Electrophoresis* **2012**, *33* (2), 316–324.

(28) Doucette, A. A.; Vieira, D. B.; Orton, D. J.; Wall, M. J. Resolubilization of precipitated intact membrane proteins with cold formic acid for analysis by mass spectrometry. *J. Proteome Res.* **2014**, *13*, 6001–6012.

(29) Puchades, M.; Westman, A.; Blennow, K.; Davidsson, P. Removal of Sodium Dodecyl Sulfate from Protein Samples Prior to Matrix-assisted Laser Desorption / ionization Mass Spectrometry. *Rapid Commun. mass Spectrom.* **1999**, *13*, 344–349.

(30) Bellei, E.; Monari, E.; Bergamini, S.; Ozben, T.; Tomasi, A. Optimizing protein recovery yield from serum samples treated with beads technology. *Electrophoresis* **2011**, *32*, 1414–1421.

(31) Candiano, G.; Dimuccio, V.; Bruschi, M.; Santucci, L.; Gusmano, R.; Righetti, P. G.; Ghiggeri, G. M. Combinatorial peptide ligand libraries for urine proteome analysis : Investigation of different elution systems. *Electrophoresis* **2009**, *30*, 2405–2411.

(32) Sharma, R.; Dill, B. D.; Chourey, K.; Shah, M.; Verberkmoes, N. C.; Hettich, R. L. Coupling a detergent lysis/cleanup methodology with intact protein fractionation for enhanced proteome characterization. *J. Proteome Res.* **2012**, *11* (12), 6008–6018.

(33) Barnidge, D. R.; Dratz, E. A.; Jesaitis, A. J.; Sunner, J. Extraction Method for Analysis of Detergent-Solubilized Bacteriorhodopsin and Hydrophobic Peptides by Electrospray Ionization Mass Spectrometry. *Anal. Biochem.* **1999**, *269*, 1–9.

(34) Whitelegge, J.; Halgand, F.; Souda, P.; Zabrouskov, V. Top-down mass spectrometry of integral membrane proteins. *Expert Rev. Proteomics* **2006**, *3* (6), 585–596.

(35) Crowell, A. M. J.; MacLellan, D. L.; Doucette, A. A. A two-stage spin cartridge for integrated protein precipitation, digestion and SDS removal in a comparative bottom-up proteomics workflow. *J. Proteomics* **2014**, *118*, 1–11.

(36) Wisniewski, J. R.; Zougman, A.; Nagaraj, N.; Mann, M. Universal sample preparation method for proteome analysis. *Nat. Methods* **2009**, *6* (5), 359–363.

(37) Wiśniewski, J. R.; Zielinska, D. F.; Mann, M. Comparison of ultrafiltration units for proteomic and N-

glycoproteomic analysis by the filter-aided sample preparation method. *Anal. Biochem.* **2011**, *410* (2), 307–309.

(38) Chen, X.; Cui, D.; Liu, C.; Li, H. Microfluidic chip for blood cell separation and collection based on crossflow filtration. *Sensors Actuators B Chem.* **2008**, *130* (1), 216–221.

(39) Modise, C. M.; Shan, H. F.; Neufeld, R. D.; Vidic, R. D. Evaluation of permeate flux rate and membrane fouling in dead-end microfiltration of primary sewage effluent. *Environ. Eng. Sci.* **2005**, *22* (4), 427–439.

(40) Hofmann, R.; Posten, C. Improvement of dead-end filtration of biopolymers with pressure electrofiltration. *Chem. Eng. Sci.* **2003**, *58* (17), 3847–3858.

(41) Holder, A.; Weik, J.; Hinrichs, J. A study of fouling during long-term fractionation of functional peptides by means of cross-flow ultrafiltration and cross-flow electro membrane filtration. *J. Memb. Sci.* **2013**, *446*, 440–448.

(42) The QIAGEN Guide to Good Microbiological Practice. Part III: Growth of E. coli cultures. *QIAGEN News* **1999**, *1*, 17–19.

(43) Arand, M.; Friedberg, T.; Oesch, F. Colorimetric quantitation of trace amounts of sodium lauryl sulfate in the presence of nucleic acids and proteins. *Anal. Biochem.* **1992**, *207* (1), 73–75.

(44) Wessel, D.; Flügge, U. I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal. Biochem.* **1984**, *138* (1), 141–143.

(45) Laemmli, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685.

(46) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **1996**, *68* (5), 850–858.

(47) Orton, D. J.; Wall, M. J.; Doucette, A. A. Dual LC-MS platform for high-throughput proteome analysis. *J. Proteome Res.* **2013**, *12* (12), 5963–5970.

(48) Orton, D. J.; Doucette, A. A. A universal, high recovery assay for protein quantitation through temperature programmed liquid chromatography (TPLC). *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2013**, *921-922*, 75–80.

(49) Vizcaino, J. A.; Csordas, A.; Del-Toro, N.; Dianes, J. A.; Griss, J.; Lavidas, I.; Mayer, G.; Perez-riverol, Y.; Reisinger, F.; Ternent, T.; et al. 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.* **2016**, *44* (November 2015), D447–D456.

(50) Huang, D. W.; Lempicki, R. A.; Sherman, B. T. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **2009**, *4* (1), 44–57.

(51) Huang, D. W.; Sherman, B. T.; Lempicki, R. A. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **2009**, *37* (1), 1–13.

(52) Kyte, J.; Doolittle, R. A Simple Method for Displaying the Hydropathic Character of a Protein. *J. Mol. Biol.* **1982**, *157* (1), 105–132.

(53) Krogh, A.; Larsson, B.; von Heijne, G.; Sonnhammer, E. L. . Predicting transmembrane protein topology with a hidden markov model: application to complete genomes. *J. Mol. Biol.* **2001**, *305* (3), 567–580.

(54) Sapay, N.; Guermeur, Y.; Deléage, G. Prediction of amphipathic in-plane membrane anchors in monotopic proteins using a SVM classifier. *BMC Bioinformatics* **2006**, *7*, 255–266.

(55) Klunk, W. E.; Pettegrew, J. W. Alzheimer's beta-amyloid protein is covalently modified when dissolved in formic acid. *J. Neurochem.* **1990**, *54* (6), 2050–2056.

(56) Beavis, R. C.; Chait, B. T. Rapid, sensitive analysis of protein mixtures by mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87* (17), 6873–6877.

(57) Zheng, S.; Doucette, A. A. Preventing N- and)-formylatino of proteins when incubated in concentrated formic acid. *Proteomics* **2016**, *16 (7)*, 1059-1068.

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