ASSESSMENT, DEVELOPMENT, AND IMPROVEMENT OF SDS DEPLETION STRATEGIES IN MASS SPECTROMETRY PROTEOMIC WORKFLOWS

by

Carolyn Kachuk

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Dedication Page

To those who raise me up

Table of Contents

List of Tables

List of Figures

Abstract

In proteomics, sodium dodecyl sulfate negatively affects trypsin digestion, chromatography, and mass spectrometry, thus, its effective depletion is critical to any protein sample workflow. A quantitative assessment of SDS depletion protocols reveals that acetone precipitation outperforms other methods in terms of SDS depletion, protein recovery, and number of protein identifications. Despite this, the need for precise pipetting challenges the reproducible success of precipitation and has prevented its wide spread use. Transmembrane electrophoresis (TME) is thus presented as a facile approach to protein purification. TME uses an electric potential to drive SDS through a dialysis membrane, while trapping protein in the sample chamber. It is amenable to both bottom-up and top-down approaches and is compatible with membrane proteins. Constant current enabled SDS depletion in 1 hour, though temperature control at constant power provides a faster rate of SDS depletion (10 min) without compromising recovery, making the device valuable to proteomic workflows.

List of Abbreviations and Symbols used

ϵ	Extinction coefficient
% T	Percent total acrylamide
$\times g$	Times gravity
1D	One dimensional
2D	Two dimensional
A	Absorbance
ABC	Ammonium bicarbonate
ACBP	Acyl-coenzyme-A-binding protein
ACN	Acetonitrile
b	Pathlength
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CA	Carbonic anhydrase
CID	Collision induced dissociation
CMC	Critical micelle concentration
CMW	Chloroform / methanol / water
cTnI	Cardiac troponin I
Cyt C	Cytochrome C
Da	Dalton
DAVID	Database for annotation, visualization, and integrated discovery
DI	Deionized water
DTT	Dithiothreitol

ECD	Electron capture dissociation
eFASP	Enhanced filter aided sample preparation
ESI	Electrospray ionization
ERLIC	Electrostatic repulsion hydrophilic interaction chromatography
ETD	Electron transfer dissociation
FASP	Filter aided sample preparation
FWHM	Full width at half maximum
GELFrEE	Gel eluted liquid fraction entrapment electrophoresis
GhxP	Guanine/hypoxanthine permease
GO	Gene ontology
GRAVY	Grand average of hydropathy
HCD	Higher energy collision dissociation
HILIC	Hydrophilic interaction chromatography
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
IAA	Iodoacetamide
i.d.	Inner diameter
ID	Identification
KDS	Potassium dodecyl sulfate
L	Lysozyme
LB	Liquid Broth
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry

LC-MS/MS	Liquid chromatography tandem mass spectrometry
LC-UV	Liquid chromatography ultra violet
LOQ	Limit of quantitation
LTQ	Linear trap quadropole
MBAS	Methylene blue active substance
MetH	Methionine synthase (zinc dependent)
MetE	Methionine synthase (zinc independent)
MS	Mass spectrometry
MW	Molecular weight
MWCO	Molecular weight cut off
Myo	Myoglobin
m/z	Mass to charge ratio
n	number of replicates
OD	Optical density
ompA	Outer membrane protein A
ompC	Outer membrane protein C
PFOA	Perfluorooctanoic acid
PSM	Peptide spectral match or Peptide spectral counts
PTB	Propensities to be buried
PTFE	Polytetrafluoroethylene
PTM	Post translational modification
R^2	Coefficient of determination
RF	Radio frequency

RP	Reverse phase
RPLC	Reverse phase liquid chromatography
RSD	Relative standard deviation
SCX	Strong cation exchange
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SISCAPA	Stable isotope standards with capture by anti-peptide antibodies
S/N	Signal to noise ratio
SPE	Solid phase extraction
SRM	Selected reaction monitoring
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TIC	Total ion chromatogram
TME	Transmembrane electrophoresis
TMHMM	Tied mixture hidden markov model
TOF	Time of flight
TPCK	Tosyl phenylalanyl chloromethyl ketone
Tris	Tris(hydroxymethyl)aminomethane
UA	Urea buffer A
Vpp	Volume of p + *p* collisions
WAX	Weak anionic exchange

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Chapter 1: Introduction *

Proteomics is the large-scale quantitative characterization of the full complement of proteins originating in a cell line, tissue, or organism.[1–5] Current research focuses on determining the diverse properties of proteins such as their abundances, modifications, and sequences.[6] Using mass spectrometry (MS), data can be acquired reliably, sensitively, and at high throughput, providing a 'snapshot in time' of how proteins exist within a biological system.[6,7] With the aid of bioinformatic platforms, distinct protein features (*e.g.* hydrophobic segments) allow proteins to be sorted into their originating cellular components (*e.g.* cytosolic, membrane, ribosomal, organelle).

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In addition, continuous improvements to MS instrumentation, sample workflows, and data analysis platforms have allowed proteomics to address important biological problems such as the discovery of disease biomarkers.[8,9] In this chapter, various aspects of proteomics will be reviewed, including bottom-up and top-down workflow strategies, the advantages and disadvantages of sodium dodecyl sulfate (SDS), the mechanisms behind SDS-protein binding, and a summary of the methods available for SDS depletion.

1.1 Proteomics

1.1.1 Proteins

Proteins are important biological macromolecules that are made up of a set of 22 amino acids arranged in a specific sequence.[10] They can act as enzymes, transporters, or provide structure to our cells.[8] To enable MS analysis, proteins must first be isolated from other constituents of our cells (*e.g.* DNA, carbohydrates). For proteins found within aqueous matrices (such as blood or urine), the homogenate is fractionated by centrifugation, resulting in a pellet high in dense cellular debris, and a supernatant high in proteins.[11] Using a technique known as differential centrifugation, the whole cell proteins within the supernatant can further be separated into membrane enriched and cytosolic enriched fractions.[11,12] For proteins found within complex matrices, such as prokaryotic or eukaryotic cells, cells are lysed before centrifugation. Following centrifugation that isolates proteins from other cellular constituents, the proteins can be purified and then identified and / or quantified by utilizing MS workflows. There are, however, numerous challenges that first need consideration.

The complexity of a proteome makes it difficult to identify all components of the sample, even with state-of-the-art mass spectrometers. Detection dynamic range is al-

ways finite and therefore low-abundance components of the sample will be more difficult to detect than the high abundance components. The plasma proteome, for instance, contains high abundant proteins (*e.g.* serum albumin at 10 mM) that overshadow / mask less abundant target proteins (*e.g.* the lupus disease biomarker interleukin 6 at 100 fM).[13–16] Other proteins can be highly hydrophobic causing aggregation and precipitation, preventing their analysis. Proteins are also temporally dynamic, with some short-lived regulatory proteins existing for as little as 0.5 hours and some long-lived nuclear pore complexes existing until the cell's death.[17,18] There are approximately 22,000 non-redundant (directly coded for by a gene) proteins in humans,[13] but protein complexity increases due to allelic variations, post-translational modifications (PTMs), alternative splicing events, and degradation, resulting in an estimated 100,000 protein forms.[7,8,19,20] When these molecular variations, which can involve more than 100 known chemical groups,[21] arise in the same protein (coded by the same gene), the resultant forms are known as proteoforms.[22] Because of the various roles that proteoforms have, their identification is crucial to understanding biological systems and facilitating biomarker discovery.[23] By understanding the function and purpose of proteins, as well as their PTMs and proteoforms, valuable information, including when and how proteins are expressed, is poised to benefit many scientific disciplines.

1.1.2 The Goals of Proteomic Studies

Proteomics continues to have significant impacts on research in many fields including medicine, biochemistry, biology, and chemistry by providing an unending source of information regarding an organism's system biology.[24] Proteoforms and PTMs in particular are very important biological molecules, and emphasis is made on the identifica-

tion of PTMs such as acetylation, glycosylation, hydroxylation, and phosphorylation.[7] When PTMs are altered, or are prevented from being formed, they can cause or be implicated in many human diseases including cardiovascular disease.[7,25–31] Consider the altered phosphorylation PTM of cardiac troponin I (cTnI), a myofilament protein that has been implicated in the pathogenesis of heart failure associated contractile disfunction.[27,31–33] Phosphorylation (a phosphate group covalently bound to an amino acid) of cTnI influences myofilament calcium sensitivity;[34–36] where a healthy heart has high levels of phosphorylated cTnI and an unhealthy heart on the verge of heart failure has no phosphorylated cTnI.[7] cTnI is therefore a standard biomarker for heart disease because it is involved in the regulation of muscular contractions and is released into the blood following cardiac injury.[31,37] The knowledge that can be attained from MS-based identifications highlight the potential of using PTMs as disease biomarkers and MS-based quantitative proteomics, which can bear a significant impact on our health.[7]

Identification of PTMs is but one goal of proteomics. Changes between proteomes of healthy and non-healthy patients can also reveal important information on the way proteins are expressed within our bodies when trying to combat disease. For example, proteomic analysis of patients with high and low levels of high-density lipoprotein show considerable differences in protein abundances for up to 380 different *m/z* species. Thus, any one of these species has the potential to be used as an early biomarker for diagnosis of cardiovascular disease.[38] Proteomics can lead to better the understanding and diagnostics of disease at early stages which will facilitate treatment, recovery, and survival.[7]

Besides diagnosing diseases, proteomics has the capability of improving treatment therapies, particularly for cancer.[39–41] With cancer being the leading cause of mortality in

both men and women from age 40-79,[42] the monitoring of cancer biomarkers is critically important for treatment. For example, the presence or absence of the oestrogen receptor, the progesterone receptor, and the human epidermal growth factor 2 receptor are used to determine the treatment regimen for breast cancer.[39–41] For tumors that contain oestrogen receptor or progesterone receptro, oestrogen inhibitors are prescribed to reduce recurrence by up to 45 % and reduce mortality by 32 %.[39] For breast tumors that overexpress human epidermal growth factor 2, antibodies or inhibitors will be prescribed to attack these receptors, and increase survival.[39,41] For patients that do not express any of these proteins, the only solution is chemotherapy.[39] To provide the best treatment, proteomics is utilized to reliably detect and quantify these proteins.[39–41]

Aside from health applications, proteomics is providing useful tools and methodologies that can be used to sequence complete proteomes. Extending such techniques to ecosystem hotspots, mass spectrometry based proteomics can gain information into the biological mechanisms underpinning the growth and success of primary producers (phytoplankton) found within the oceans. The measurement of methionine synthase proteins (MetH and MetE) in phytoplankton can be used to evaluate the availability of vitamin B_{12} in the oceans.[43,44] Since phytoplankton cannot produce B_{12} on their own, the presence of MetH indicates high abundances of B_{12}, and the presence of MetE indicates low abundance of B_{12}. When there is a high abundance of B_{12}, the growth of phytoplankton is not limited. As primary producers, marine microbial organisms are responsible for producing half of the oxygen on earth as well as the synthesis of organic carbon, which is fundamental to the entire marine ecosystem as well as the global climate. This is a timely and important topic, as ocean ecosystems are facing a time of rapid change,[45] with poten-

tially drastic implications for humans ranging from changing the magnitude of the ocean carbon sink and increasing the negative impacts of harmful algal blooms.[46,47]

To achieve these goals, proteomic assays need to be both sensitive and specific for individual proteins. Thus an additional goal of proteomic studies (and a primary focus of this thesis) is to increase sensitivity and specificity of analytical detection methods to accurately and reliably measure proteins, particularly those in low concentration, from a small sample volume.[13] One way to increase specificity is to combine stable isotope standards with capture by anti-peptide antibodies (SISCAPA),[48] but this method cannot detect proteins of low abundance.[13] Multiplexed immunoassays have the sensitivity to detect these low abundance proteins, but there can be high cost ($50 k – 100 k) and significant time requirements (~ one year) associated with method development,[49–52] making it impractical for anything but high-value targeted proteomics.[13] Selected reaction monitoring (SRM) analysis by MS has the potential to replace immunoassays for sensitive quantitative detection of low abundant proteins, but improvements to ionization, ion transmission, ion separation, signal-to-noise ratio, MS resolving power, and front-end sample processing strategies are still required.[49]

1.2 Mass Spectrometry of Proteins

1.2.1 Bottom-Up Mass Spectrometry

Bottom-up mass spectrometry sequences peptides to identify proteins. Peptides are proteins cleaved into smaller fragments. Cleavage is typically done by enzymatic digestion (*e.g.* trypsin or pepsin), but can also be done with acids, CNBr, or light.[53,54] Digestion is commonly done following protein separation strategies such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), though separation prior to di-

gestion is not necessary. Once the peptides are generated, electrospray ionization (ESI) is typically used to introduce ions to the mass spectrometer.[55,56] ESI is a 'soft' ionization technique that directly ionizes samples from solutions and, because it is readily carried out with mixed aqueous-organic solutions,[57] it can be coupled to liquid chromatography (LC). By combining LC with ESI-MS (LC-MS), peptides can be separated with chromatography prior to reaching the spectrometer, thus allowing for greater protein identifications by reducing the load on the system. Inclusion of nanospray ESI, which reduces the flow rate of the system, also enhances the detection sensitivity of proteins, facilitating their detection.

Today's ESI LC-MS systems commonly employ time-of-flight (TOF),[58] orbitrap,[59,60] linear ion traps,[61] or quadrupole mass spectrometers.[6,62,63] To characterize a given peptide, specific ions are isolated in MS mode based on their *m/z* ratio and further fragmented, resulting in tandem mass spectrometry (LC-MS/MS) spectra.[6] The MS/MS spectra provide sequence information for a specific list of proteolytic peptides, often several for a given protein. The data are interpreted by matching the fragment ions to the theoretical fragment masses as calculated from the same proteolytic digestion of a predetermined protein sequence database. Success is dependent on the number of peptides identified and the mass accuracy of the spectrometer.

The number of peptides identified is influenced by the type of method used to generate fragment ions. Fragmentation can be achieved by methods such as higher energy collision dissociation (HCD),[64,65] electron capture dissociation (ECD),[66] or electron transfer dissociation (ETD),[67,68] though it is collision induced dissociation (CID)[69–71] that is typically used with bottom up MS. The resultant MS/MS scans are recorded and the

fragmentation pattern of fragments containing the N-terminus (b ion) and those containing the C-terminus (y ion) are used to calculate the mass differences between the ions and sequence the proteolytic peptide.[72] Though there is similar homology between many proteins, their cleavage results in a set of unique fragments that are analogous to a fingerprint.[9] By searching these fingerprint sequences against databases (SEQUEST / MASCOT)[73,74] with known sequences derived from DNA sequences, protein identifications can be made.

Bottom up analysis is the most widely used MS-based protein identification approach. The greatest advantage of bottom-up is that when cleaving a protein, there will be at least one peptide that is readily detected by MS. This peptide is highly reproducible for a given protein and is known as a proteotypic peptide. By searching against proteotypic peptide libraries, the originating protein can be determined, and thus bottom-up analysis essentially 'guarantees' protein identification.[75] In addition, many 'older' (> 10 years old) mass spectrometers have resolutions that limit the charge states that can be reliably determined, which limits the size of the molecule that can be introduced to the mass spectrometer. With the bottom-up approach, this molecular weight / size restriction is a non-issue. Han *et al* and Zhang *et al* provide useful summaries of the performance characteristics of commonly used mass spectrometers.[19,76]

The down-side of the bottom-up approach is that it only achieves 40 – 90 % sequence coverage because of incomplete recovery of peptides following digestion.[9,77–79] The identification of PTMs, which are present in low abundances, is also unlikely unless a targeted approach is used.[80] This can be a significant concern when considering that the discovery of PTMs and their alterations have a significant role in the prevention, diagno-

sis, and treatment of diseases. The information that can be obtained from multiple PTMs on a given protein is also lost because of digestion. To reconstruct all the peptide fragments to reconstruct the original PTM is challenging, particularly when you are working with a complex protein mixture. Considering that the prokaryote *Escherichia coli* (*E. coli*) is considered a complex proteome, reconstructing a eukaryotic proteome from the peptides incurred from the bottom-up approach is even more challenging. Eukaryotic cells, particularly mammalian cells, have a greater number of proteins, longer sequences, wider concentration ranges, and greater variability with modifications and alternative splicing events. Thus, for bottom-up to be able to characterize whole proteomes of these cells, improvements to the accuracy, sensitivity, quantitation, and identification of proteoforms and PTMs is needed.[81]

1.2.2 Top-Down Mass Spectrometry

In contrast to bottom-up MS-based protein identification, the top-down approach introduces proteins into the mass spectrometer without enzymatic cleavage. The information that can be attained from top-down MS-based protein identification is remarkable, particularly concerning PTMs and proteoforms, though new technological developments, particularly in front-end sample preparation strategies, are still needed to advance the top-down approach to match the detection performance of bottom-up MS.[7,21,82–84] Toby *et al* provide a comprehensive review of the current status of top-down proteomics and its application to proteoform and PTM identifications.[85] The diverse range of proteins, PTM's, and proteoforms that exist create challenges in their analysis associated with solubility, separation, abundance, complexity, and molecular weight (MW).[7,21]

Complex mixtures of proteins are challenging to assay by MS as there are multiple charge states and a large number of isotopic peaks that mask the signals of larger proteins.[21] Separation techniques can help, but prior protein separation by chromatography has several challenges[86] as proteins tend to interact in undesirable ways with the chromatographic stationary phases.[87–93] Ionic interactions are common, resulting in adsorption of the protein,[94] shifts in retention time,[95] peak tailing or asymmetry,[96] and changes to the 3D structure of the protein.[88,97] Non-binding electrostatic interactions can also occur, resulting in "ion-exclusion" that prevents the proteins from interacting with the pores of the column, thus eluting sooner than predicted.[89] Hydrophobic interactions can also play a role, leading to increased retention or on-column denaturing.[98] Alternatives to chromatography, such as separation by SDS-PAGE, are also not suitable for top-down workflows as they require digestion to recover the entrapped proteins. A solution to the top-down separation challenge is gel-eluted liquid fraction entrapment electrophoresis (GELFrEE),[99] wherein proteins are separated by molecular weight and collected as aqueous fractions in a SDS / Tris(hydroxymethyl)aminomethane (Tris) / Glycine buffer. Though utilizing GELFrEE[99] addresses the issue of reduced separation, it introduces SDS to the matrix. As will be discussed in Section 1.3.2, SDS is detrimental to down-stream analysis by mass spectrometry and needs to be removed.

With advances in mass spectrometry technologies, as well as the speed and resolution in MS/MS fragmentation, proteins as large as 200 kDa can now be characterized using top-down strategies.[77] Despite the fact that top-down does not yet identify as many proteins as bottom-up, it is beginning to emerge as the preferential approach to MS-based protein identification.[84] This is mainly because it provides 100 % sequence coverage of

intact proteins,[21] including PTMs, proteoforms, and sequence variations.[7,84,85] This is possible because, in contrast to using HCD or CID to fragment ions (which cleave bonds at the lowest activation energy)[82] top-down MS typically uses non-ergodic techniques such as ECD and ETD that cleave along the protein backbone.[82,100] These non-ergodic fragmentation techniques preserve labile PTMs, which allows quantification of proteoforms,[101] mapping of modifications with full sequence coverage, discovery of unexpected modifications, identification of positional isomers, and the determination of the order of multiple modifications.[76,82,102] Any discrepancy between the exact mass obtained by top-down MS and the predicted MW calculated from the DNA sequence is attributed to a modification.[77] The type and location of the modification is obtained by corresponding discrepancies in fragment ion masses.[21,100,103–105] Though there is a reduced complexity in terms of number of individual species present in the sample that is being introduced to the mass spectrometer,[7] top-down MS encounters significant limitations because of challenges with protein fractionation, ionization, and fragmentation.[19] The top-down approach has recently been successful in identifying potential tumor biomarkers from breast cancer xenografts[84] and from salivary glands.[106]

1.2.3 Bioinformatic / Classification Software

With the vast amount of data that is obtained through mass spectrometry of proteins, bioinformatics has become an essential part of any proteomic workflow.[107–119] Online bioinformatic platforms such as DAVID (Database for Annotation, Visualization, and Integrated Discovery)[120–122] provide functional annotation tools, particularly the discovery of biological themes, using Gene Ontology (GO) terms.[123,124] Gene Ontology is a consortium whereby GO terms provide a controlled vocabulary for representing and de-

fining concepts and classes that describe gene functions along three aspects: molecular function, cellular component, or biological processes. These terms are then used in functional annotation tools to describe the biological identity (*i.e.* its molecular function, biological roles, and subcellular location).[125] Using the gene functional classification tool and the functional annotation clustering tool developed by Huang *et al,*[126] DAVID enables large protein lists to be organized and condensed into meaningful categories such as ribosomal, membrane, and cytosolic proteins.

Another useful tool in the classification of proteins relates to hydrophobicity. Using Grand Average of Hydropathy (GRAVY),[127] numerical scores are assigned to every amino acid (hydrophilic are < 0). The higher the GRAVY score, the more hydrophobic proteins tend to be, which suggests that they are likely associated with the membrane. Membrane protein topology can then be predicted using algorithms such as TMHMM (Tied Mixture Hidden Markov Model)[108] and AmphipaSeek[107], which identify transmembrane helices and in-plane membrane anchors, respectively, within protein sequences. These types of bio-informatic tools are important for detecting remote sequence homologies,[110] sub-cellular localization,[128] and protein-protein interactions.[129]

1.3 The Role of SDS

1.3.1 SDS Benefits

SDS-PAGE (one dimensional or two dimensional) is undoubtedly the most widely used analytical method to separate, display, and characterize proteome samples. Prior to the use of SDS, the separation of proteins was achieved through a starch or polyacrylamide matrix that restricted separation as a function of isoelectric point (pI).[130–132] This quickly was determined to be undesirable as many proteins have similar pIs and

could not be sufficiently resolved for further study.[133] Though the first use of SDS with the separation of proteins is disputed and occurred somewhere between 1963 and 1965, the conclusions that SDS could be used to successfully dissociate proteins from complex matrices was the same.[134–136] Following this discovery, SDS was utilized in a series of papers to dissociate proteins from poliovirus, adenovirus, and influenza, solidifying its utility in proteomic studies.[134,137,138] Further research confirmed its utility for separating proteins by molecular weight.[139,140] As MS-based approaches for protein characterization were not in existence at this time, Edman degradation protocols and electroelution / electroblotting techniques were commonly used for protein sequencing, which heavily relied on SDS-PAGE separation.[141–143] With the rapid increase in the size of sequence databases, the Edman degradation protocol became too slow and had relatively poor sensitivity.[6] The breakthrough in soft ionization techniques (*e.g.* ESI) allowed mass spectrometry to replace Edman degradation, but the need for SDS-PAGE separation remains. Today, SDS is still extensively used with mass based protein separation techniques such as SDS-PAGE[139,144] and GELFrEE[99] prior to MS analysis. Clearly SDS plays an important role in effective protein separation, but this is not the only reason that SDS is used in proteomics.

Another important aspect of SDS in proteomics is the role it plays in cell lysis and the denaturation and solubilisation of proteins. This is particularly useful for the analysis of membrane proteins, which are normally challenged by their hydrophobic character. Though they account for nearly 30 % of the proteins produced by genomic sequences,[145] membrane proteins have low levels of expression and decreased solubility and digestion efficiency relative to their hydrophilic counterparts.[146] Consequently, these important

medicinal targets are often underrepresented in proteome analysis platforms.[147–149] The use of SDS remedies this issue as it dissociates lipid membranes and solubilizes membrane proteins. SDS is an amphipathic synthetic surface active agent (surfactant) where high concentrations (between 1 and 4%) enhance protein extraction and solubilisation from cell lysates.[148,150–154] The hydrophobic region of SDS effectively denatures proteins including misfolded, precipitated, and hydrophobic proteins.[155–157] In certain circumstances (generally at low surfactant concentration), SDS can even enhance MS signals[158] and prevent adsorption onto container walls.[159] Unfortunately, SDS is undesirable for downstream protein analysis, and is further considered notoriously difficult to deplete.[160,161]

1.3.2 SDS Detriments

1.3.2.1 SDS and Chromatography

Above 0.01 %, residual SDS is incompatible with reversed-phase liquid chromatography (RPLC) and interferes with binding and elution during chromatography separation.[162–165] When SDS is present (> 0.05 %), the elution of peptides from a C18 column occurs significantly later and with reduced separation and enhanced peak broadening.[163,166] Above 0.02 % SDS there is significant broadening of the peak,[164,167,168] and with a concentration of 0.1 % all proteins will elute from the column as a single broad peak.[163] The reduced separation and peak broadening effect that SDS has on chromatography is related to the number of arginine and lysine residues present in the sequence.[163] There are two proposed binding interactions that caused this change in chromatography: (1) the anionic detergent binds to the positively charged arginine and lysine residues, rendering the peptide more hydrophobic and therefore increases the elution

time, or (2) the anionic detergent binds to the column matrix, effectively converting the C18 medium into a negatively charged cation-exchange matrix.[163] This can result in complete retention of SDS bound peptides on the C18 column[155] or it can cause peak shifts.[164] Regardless of the binding interaction, SDS negatively affects chromatographic separation prior to mass spectrometry.

1.3.2.2 SDS and Mass Spectrometry

Residual SDS will also suppress ESI MS signals by altering the surface tension of ESI microdroplets.[164,169] LC-MS generally tolerates up to 0.01 % SDS (100 ppm),[164] though some signal deterioration may still be apparent at this level, including formation of SDS adducts in the MS spectrum.[170] At 1 % surfactant concentration, regardless of whether the detergent is cationic, zwitterionic, anionic, or non-ionic, no signals are detected.[171] When assessing SDS's compatibility with mass spectrometry, there are four effects that should be considered: (1) surfactant background ions that interfere / obscure the protein signal, (2) suppression of the protein signal, (3) adduct formation, and (4) shifts in charge envelope.[171] Above 0.01%, SDS clusters dominate the mass spectra[169] and suppress the signal intensity of ESI mass spectra[172,173] to less than 10 % of the original intensity.[169] At these concentrations protein-SDS adducts are observed in the spectra,[57,171,174] leading to a substantial shift of charge distribution and overlap of protonated molecular ions.[57] Charged Na^+-attached SDS monomers can also be observed extending to high *m/z*.[169,171] Even at low SDS concentrations, SDS adducts are still present. Thus, SDS is considered non-compatible with ESI-MS, requiring its removal prior to analysis.

1.3.2.3 SDS and Trypsin Digestion Efficiency

Trypsin is a water soluble serine protease found within the pancreas of vertebrates. It functions as a digestive enzyme aiding in the breakdown of lipids and carbohydrates. An imbalance of trypsin and antitrypsin in the pancreas causes pancreatitis, whereby trypsin will start to attack the pancreatic tissue.[175,176] The trypsin molecule contains two domains that each contain an antiparallel beta sheet.[177] As discussed below in Section 1.3.4, the presence of beta sheets reduces the ability of SDS to unfold the protein, but at 0.08 % (3 mM) SDS, trypsin is unfolded.[178] In agreement with this, studies have shown reduced trypsin activity when residual SDS concentrations are above 0.1%.[148,156,179,180] Above 1 % trypsin digestion completely stops.[159] This is caused by the denaturation of the enzyme and / or interference with the protein-enzyme complex.[159] Even with 0.01 % SDS present during digestion, the MS signal is suppressed to half of what was obtained without any SDS.[181] Thus, SDS must be removed to effectively digest proteins prior to bottom-up MS anlaysis.

1.3.3 SDS – Protein Binding

To understand the relationship between SDS and protein, an understanding of the thermodynamic properties of SDS-protein is needed. There are two modes of SDS-protein binding. One is a strong interaction (specific, hydrophilic binding), and the other is a weak interaction (non-specific, hydrophobic binding).[182] The majority of binding between SDS and protein, however, is relatively non-specific, occurring in a wide variety of proteins of different moieties.[183] Nielsen *et al*[184] give a comprehensive table of the thermodynamic and stoichiometric data for the binding of SDS to bovine serum albumin (BSA) at 25°C and also provide indications that SDS thermally stabilizes BSA.[184–186]

With BSA, SDS has two classes of binding regions that contain thermodynamically similar sites, termed high and low affinity sites. The high affinity region has a binding constant of 3.3×10^7 M^{-1}, a Gibbs free energy of -42.9 kJ/mol, and a binding enthalpy of -35.5 kJ/mol. The low affinity region has a binding constant of 1.9×10^5 M^{-1}, a Gibbs free energy of -30.1 kJ/mol, and a binding enthalpy of -28.1 kJ/mol.[184–192] A higher value for the binding constant at the high affinity region indicates that this is where the strong 'bound' interactions between SDS and BSA occurs that is driven by Coulombic (electrostatic) forces. Considering that the Gibbs free energy change from an aqueous solution to pure alcohol (1-dodecanol) is -26.5 kJ/mol, the Gibbs free energy in the low affinity region may, at higher SDS concentrations, be solely driven by a hydrophobic mode of interaction.[184] This non-specific weak hydrophobic binding may exceed up to 100 surfactant molecules per protein molecule.[184] The dominant factor in the binding enthalpies of the high and low binding regions is caused by specific ionic interactions of the surfactant sulfate ion on the head group to the amino side chains, which may account for 25 – 35 kJ/mol of the measured binding enthalpy, or -2.5 kcal / mol / residue.[184,193,194] Thus, taking into consideration the thermodynamic properties of SDS-protein binding at the high and low affinity binding regions, SDS-protein interactions can be further understood.

1.3.4 SDS – Protein Interactions

SDS is anionic and binds firmly to protein / peptide chains through a variety of physiochemical mechanisms (ionic, hydrophobic, electrostatic).[182,184,195–197] These binding mechanisms cause effective denaturation by altering the secondary and tertiary structures of protein.[198] To understand these mechanisms, consideration must first be given to how SDS interacts with itself. In water, when SDS reaches a concentration greater than

the critical micelle concentration (CMC) of 7 – 8.2 mM (~ 0.2 %), monomers begin to form micelles, a circular aggregation of molecules as shown in Figure 1-1.[191,199–203] Upon micelle formation, hydrophilic sulfate head groups create an outer shell that protects the hydrophobic acyl chains as they aggregate to the center. Data indicates that 10 out of the 12 acyl segments become dehydrated during this process, stabilizing the structure.[184] Hydration of the upper few segments is likely caused by the charge repulsion between the sulfate head groups leaving a loosely packed micelle-water interface.[184] Above the CMC each new SDS molecule is incorporated into the micelle,[190] which has an average radius of 18.5 ± 1 Å.[204]

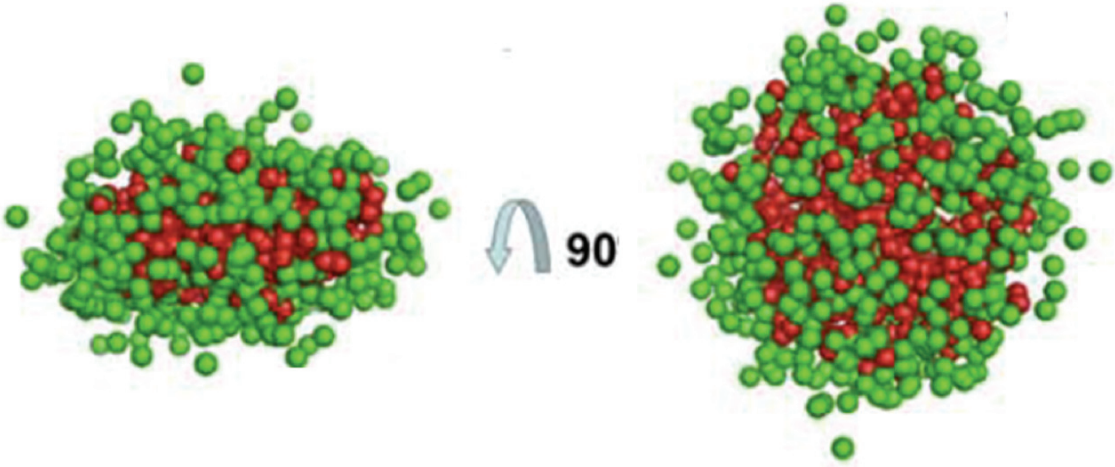

Figure 1-1. Pure SDS elliptical micelle structures fit from small angle X-ray scattering and indirect Fourier transform data. The structures of the micelles are shown as spheres at the position of the Monte Carlo points. The hydrocarbon tails are represented by red spheres, whereas headgroups and counterions are represented by green spheres. Five hundred points are used for each of the two contributions. Figure reprinted with permission from reference 198. Copyright Elsevier 2009.

The critical micelle concentration is not a constant and changes in conjunction with the sample matrix.[182] Its value can be determined by measuring changes in enthalpy

as described by Kelley and McClements.[205] Generally, the CMC is proportional with temperature and inversely proportional with ionic strength.[182,199,200,206,207] As the CMC increases, so does the SDS monomer concentration,[190] which proportionally influences the binding ratio to protein.[190] Above the SDS-monomer concentration of 8×10^{-4} M, the equilibrium SDS binding ratio is 1.4 g of SDS per 1 g of protein.[190,208] Though often cited as a constant, binding ratios have been reported to vary from 0.4 to 2.2 g of SDS per gram of protein, in correlation with binding to high and low affinity regions, respectively.[183,209–213] Variation in the binding ratio is caused by: (1) the type of protein, where the presence of disulfide bonds has significant influence,[183] (2) a change in the charge of the protein, where a normally charged protein typically binds between 1.3 and 1.7 g SDS per g of protein,[211] or (3) an increase in salt concentration.[183] Changes in these parameters can alter the binding ratio by greater than 50 %.[183,211] Typically there are two SDS-binding plateaus that occur; one at 0.4 g SDS per g of protein, and another at 1.4 g SDS per g of protein.[190] These plateaus are related to how SDS denatures protein and is discussed below.

A classic model of SDS-protein denaturation was proposed by Tanford,[157] and confirmed by Turro and Lei[201] and Andersen *et al.*[198] In this model, SDS, which has formed ellipsoid of revolution micelles,[198] first binds to high affinity regions (0.4 g SDS / g protein), which induces little structural change. This is followed by a plateau, then a massive uptake in SDS at low affinity regions that causes denaturation (1.4 g SDS / g protein). Andersen *et al* proposed that, when considering the interaction of SDS with bovine acyl-coenzyme-A-binding protein (ACBP),[198] micelles containing 16 individual SDS molecules begin to form at SDS concentrations ≥ 1.3 mM and, as SDS approaches

2.8 mM, the micelles grow to contain 33 molecules. It is at this stage where SDS begins to denature the protein and the tertiary structure is lost. Above this concentration (up to 5.2 mM), the micelles continue to grow to contain 42 molecules, but there are no additional changes to the secondary structure. At concentrations > 5.2 mM SDS, micelles grow to a maximum of 60 SDS molecules and rotational movement is reduced. Figure 1-2 shows a representation of these events, which result in the proposed bead-on-a-string, "necklace" model[201,209,210,212] where denatured / solubilized proteins are essentially encased in an SDS micelle shell.[156] The saturation of binding is reached when there is ~ 1 SDS molecule per 2 amino acid residues.[214] The specifics of this proposed model (*i.e.* the number of SDS monomers at a given stage) can change depending on the protein that SDS is interacting with.

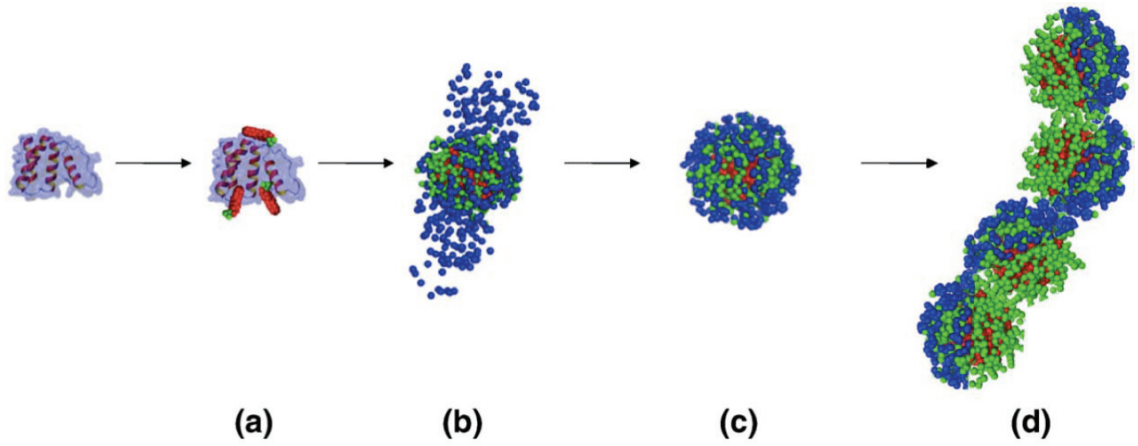

Figure 1-2. Schematic representation of the different stages of ACBP denaturation. Green points represent the head group of SDS. Red points represent the hydrocarbon tails of SDS. Blue points represent protein distribution. In stage A, ACBP binds between 1 and 3 SDS molecules without losing the native structure. Stage B involves the formation of a decorated micelle of 37 SDS molecules that binds 2 ABCP molecules. Further binding of SDS to a total of 40 in stage C leads to monomeric ACBP with a shell-like structure of SDS. The structure presented in stage D is speculative, but it represents the "beads-on-a-string" model that has been proposed for protein interactions with SDS micelles above the CMC. Figure reprinted with permission from reference 198. Copyright Elsevier 2009.

The way SDS – protein interactions occur varies depending on the protein, temperature, pH, and ionic strength.[205,215] When considering the type of protein, there can be multiple binding steps involved or binding can be fairly direct.[184,205,216] The amount of time for SDS to fully equilibrate with protein can also vary considerably and has been recorded to take anywhere from 3 hours up to several days for maximal binding.[211] This 'resistance' to binding SDS is not linked to primary structure[217] or thermodynamic stability,[211] but is correlated to SDS's ability to unfold the protein[183] and to the presence of high amounts of β-sheets in the protein.[218] These β-sheets unfold slowly,[217,219] sterically blocking SDS access. This is in contrast to α-helices, which have local stabilizing interactions that facilitate solubilisation by SDS micelles.[213,215] These characteristics of SDS to effectively and efficiently bind protein to denature and solubilize it are what makes SDS the preferred detergent for proteomics.

When considering the removal of SDS from protein, the physical properties of the detergent,[220] as well as those of the proteins that bind it, must be considered and the binding energies that hold them together must be overcome. Effective protocols to deplete unbound and bound SDS are an integral part of every proteomic workflow and the success of removing the detergent can be achieved by considering these parameters.

1.4 Solutions to SDS Problems

1.4.1 SDS Alternatives

There are several alternatives to using SDS in proteomic workflows such as: (1) perfluoroacetic acid (PFOA), which does not solubilize membrane proteins as effectively as SDS and requires a multistep evaporation strategy to remove prior to bottom-up

MS analysis;[221] (2) 60 % methanol, which also does not solubilize membrane proteins as effectively as SDS but can be present in higher concentrations during digestion;[156,222] (3) chaotropic agents, which denatures proteins[223] but ultimately lowers the digestion efficiency of trypsin greater than SDS;[224] (4) non-ionic and cationic surfactants, which are slow at denaturing protein;[213,215,225–227] (5) zwitterionic surfactants, which destabilize protein;[228,229] (6) acid-cleavable surfactants, which can enhance digestion but have lower solubilisation efficiencies than SDS;[180,230] (7) phase-transfer surfactants, which require enhancement for effective solubilization.[231]

These approaches have been reviewed thoroughly elsewhere,[148,149,213] but are often less effective than SDS at solubilizing the proteome, work under limited conditions,[231] or may even be incompatible with LC-MS or digestion.[165] Therefore, SDS remains the preferred choice among researchers, elucidating the need to remove SDS prior to protein digestion and analysis by electrospray ionization (ESI)[55] mass spectrometry.[232,233]

1.4.2 SDS Removal

1.4.2.1 Electrophoresis

SDS is considered to be quite difficult to remove, yet there is a method that utilizes SDS and is compatible with mass spectrometry.[234–236] SDS-PAGE is a gel-based electrophoretic strategy that retains protein in a polyacrylamide matrix, facilitating in-gel digestion following detergent removal.[237,238] Proteins trapped within the gel are stained to visualize the bands, which are then excised from the gel with a scalpel. Removal of any residual SDS occurs through a series of ammonium bicarbonate and acetonitrile washes, which also dries the gel.[239,240] This approach is very effective at removing SDS

(> 99.99 % depletion).[237] However, gel-based methods have limitations when it comes to identifying proteins.[241] These limitations include difficulties in extracting intact proteins, deeply entrapped peptides, and restricted access of proteases that leads to low cleavage yield and low recovery of large or highly hydrophobic peptides.[242,243] Soffientini and Bachi[244] present a novel in-gel digestion process called STAGE-diging. It is reminiscent of the in-gel digestion method presented by Shevchenko *et al*[235], only this new protocol takes place in a pipette tip with C18 plugs, as opposed to an Eppendorf vial. They do this without compromising the rate of protein identification or quantitation.[244]

The tube gel digestion protocol introduced by Lu and Zhu[173] also incorporates in-gel digestion, but without gel separation. It was designed for use with membrane proteins, but can be used with any protein type. The method uses a high concentration of SDS (5 %) to solubilize and denature the proteins before trapping them, dispersed, within an acrylamide gel. The detergent is then washed out, and the proteins enzymatically digested. Though they do not quantify how much SDS is removed, they report that the tube gel removes enough detergent so as not to effect chromatography. The benefits of this method is the ability to utilize high SDS concentrations to solubilize the sample and that it can be used with protein concentrations as low as 10 fmol. The downside of this method is that there is no separation of proteins within the tube gel, and separation is critical before MS analysis.

1.4.2.2 Dialysis

Dialysis is the classic approach to protein purification where SDS monomers pass through dialysis tubing by passive diffusion. As the concentration of SDS is depleted from the protein solution to below the CMC, all unbound SDS micelles disperse back in-

to monomers, further allowing SDS depletion.[245] This is only effective at removing unbound SDS though and SDS that is bound directly to protein is not depleted. This is because passive diffusion is unable to overcome the free energy of binding between SDS and protein. In fact, most SDS-protein binding ratios have been determined by using dialysis.[183] Though SDS-protein binding ratios vary depending on the protein and the sample matrix, it typically equates an average of 1.4 g of SDS per 1 g of protein. Given that dialysis only removes unbound SDS,[182] and that it takes a long time (up to 96 hrs),[246] it is insufficient for SDS depletion ahead of MS analysis.[247,248]

1.4.2.3 Column-based

Column-based SDS depletion strategies have also been employed to deplete SDS from protein samples.[237] The acronym hydrophilic interaction liquid chromatography (HILIC) was first introduced in 1990,[249] though the separation technique has been in use 1975.[250] It is particularly useful in the separation of polar compounds that do not bind to reversed phase (RP) materials.[251] When the stationary phase adsorbs water, it becomes hydrophilic and polar peptides partition into the aqueous layer.[252] HILIC separates peptides based on their hydrophilic interactions with an ionic resin.[19] With a variety of resins to choose from,[253] it is very useful for peptide fractionation and PTM analysis.[254] Using an organic to aqueous gradient inverts the peptide retention order. The use of volatile organic phases in HILIC provide an increase in ESI-MS sensitivity.[251] A specific form of HILIC, called ERLIC (electrostatic repulsion hydrophilic interaction chromatography), uses a weak anion exchange resin (WAX) to further separate ions based on isoelectric point (pI).[255] Only a few works have incorporated HILIC in regular sample preparation.[256] Jeno *et al*[257] use HILIC to successfully remove SDS by trace enrichment

prior to digestion, though they do not quantify it. Trace enrichment was done by loading proteins with up to 200 µg of SDS per injection onto a column that was equilibrated with 70 % propanol / 50 mM formic acid. The protein was eluted with a decreasing concentration of propanol and showed no traces of residual SDS.[257]

Ion exchange chromatography[258,259] is based on the charge differences between the protein and the micelle.[259] Han *et al*[260] utilize these principles to effectively remove residual SDS with strong cation-exchange chromatography (SCX) prior to HPLC MS, but did not quantify its effectiveness. SCX peptides and proteins that are positively charged at low pH are retained on a negatively charged stationary phase (phospho or sulfo groups). A disadvantage of SCX chromatography is that similarly charged peptides elute within a narrow time-frame.[254] Alternatively, anion exchange can also be used where peptides and proteins are separated as negatively charged species at high pH interacting with a positively charged stationary phase. With reports of SCX removing up to 99.99 % of SDS,[261] sample losses occur when SDS-peptide complexes bind irreversibly to the column.[262,263]

Reversed phase liquid chromatography (RPLC) has also been employed to separate SDS from protein while retaining > 90 % of protein.[264,265] Peptides or proteins are separated based on hydrophobicity and their interactions with C4, C8, or C18 alkyl chains, eluted with low to high organic solvent gradients. Under appropriate conditions, greater than 99.9 % of SDS can be removed.[264] Parameters that affect the success of SDS depletion include column length, inclusion of 8 M urea, type of protein, and SDS-Protein binding interactions.[264] Several authors have reported that SDS interferes with RPLC by

binding to the stationary phase and to the peptides.[163,166] RPLC is therefore the same as the others and only effective at removing unbound SDS.

Size exclusion chromatography (SEC) [266] is where peptides or proteins are separated based on their size in solution using a non-interactive stationary phase with uniformly sized pores. Molecules pass through in order of decreasing hydrodynamic volume (*i.e.* size).[88] Like other column based methods, there are reports of protein loss when SDS is present in the sample.[267] This is likely due to SDS being trapped within the pores of the column while still binding protein, thus preventing its flow through. A recent publication by Lambrecht *et al* cautions that even though SEC is supposed to utilize a non-interactive stationary phase, there are actually quite a few non-size interactions that occur, particularly when SDS is present.[268]

Although there are many types of column based techniques available, none are able to effectively deplete bound SDS. Bound SDS can lead to protein loss by irreversibly binding to the column stationary phase, or by allowing the bound protein to not be retained on the column in the first place. Thus column-based methods are not an ideal approach to SDS removal.

1.4.2.4 Filtration

Filtration is a technique that attempts to disrupt the SDS-protein binding. Commercially available regenerated cellulose spin molecular weight cut off (MWCO) filters can be used to retain the larger protein molecules while allowing SDS to spin through.[153,269] Manza *et al*[269] use them to reduce the SDS to levels compatible with tryptic digestion (0.1%) and LC-MS/MS analysis (0.01%), though they do not quantify the concentration of residual SDS. They reported little difference between 3000 or 5000

MWCO filters, regardless of brand, though acknowledge that using larger MWCO filters (20 – 30 kDa) would result in loss of smaller proteins and peptides.[269]

Wisniewski *et al*[153] incorporate larger MWCO cellulose filters (10 or 30 kDa) in their filter aided sample preparation (FASP) protocol and replace the ammonium bicarbonate wash with an 8 M urea wash.[153] This was considered a breakthrough paper and has been cited 1169 times at the time of writing. This is mainly because it highlighted the benefits of incorporating SDS into the proteomics workflow. The urea wash they incorporated dissociated the SDS from the proteins and reduced the detergent concentration to levels below its CMC, facilitating its removal.[151,223,264,270] They were able to employ a high concentration (4 %) of SDS to solubilize their proteins, which allowed for many more types of proteins to be detected (*e.g.* membrane proteins and low abundance proteins).

This FASP protocol developed by Wisniewski *et al*[153] does remove greater than 99.99 % SDS,[237] but unfortunately upwards of 50% protein loss can be expected,[237,271–273] owing to nonspecific binding or incomplete digestion.[147] FASP also involves a considerable number of steps, requiring significant time to complete. Improvements to FASP have been tried in an attempt to increase recovery. Erde *et al*[151] include the use of 0.2 % deoxycholate acid in the digestion ammonium bicarbonate solution. Doing this, they identified 284 proteins using their method (eFASP) *vs* 52 proteins by Wisniewski *et al*'s method (FASP), but they too do not quantify SDS depletion or protein recovery. Though recovery remains poor with these methods, they do highlight the benefits of incorporating SDS into proteomic workflows, particularly in regards to solubilisation and membrane protein recovery.

1.4.2.5 Precipitation

In Section 1.4.2.1, the use of acetonitrile was discussed as a way to dry the SDS-PAGE gel, which effectively traps protein within the pores and washes SDS away. The reason the protein becomes trapped, as opposed to also washing away, is that protein precipitates in the presence of organic solvents. Using solvent precipitation reduces the hydrophobic SDS-protein binding interactions. There are two types of precipitation methods. One is where the contaminant (detergent) can be precipitated. Zhou *et al*[274] use KCl to remove SDS by making the precipitate potassium dodecyl sulfate (KDS). This method works very well with unbound SDS but is unable to fully remove the bound SDS.

Precipitation can remove the unbound and bound SDS when the precipitate is the target analyte (protein).[12,164,237] Precipitation using a heptane mixture[160,163] and ethanol precipitation[275] are methods that have shown promising recoveries (> 95 %) and SDS depletions to 0.001 %. Chloroform-methanol-water (CMW) and acetone precipitation are popular precipitation methods where 1 – 2 % SDS can be effectively removed to levels compatible with MS analysis.[164,276] Acetone precipitation typically has the lowest loss of proteins / high precipitation efficiency compared to CMW.[164,237,276–280] There have been conflicting reports on the effectiveness of acetone precipitation protein recovery where the loss of protein can easily occur, particularly at concentrations < 5 μg, whereby the protein pellet is difficult to visualize in the Eppendorf vial.[164] This can be alleviated by adding more washing steps, whereby a larger volume of supernatant can be left in the vial to evaporate off (two washes = 0.001% SDS),[164] and by carefully carrying out pipetting / sample manipulation techniques.[164,237]

A disadvantage of precipitation is that once the detergent is removed, protein pellets need to solubilized in a MS compatible solvent prior to LC-MS analysis.[257] This can be difficult, particularly for membrane proteins. Concentrated urea (8 M) can be used, as can sonication in aqueous solvents. However both of these methods are ineffective at solubilizing all proteins.[281] Doucette *et al* have remedied this problem by demonstrated high yields (> 80%) through acetone precipitation of membrane proteins using cold formic acid to solubilize the protein pellet.[12]

To negate the need for precise pipetting and to ensure high protein purity with reproducible yields, Crowell *et al*[282] developed a two-stage filtration and extraction cartridge (ProTrap XG) to facilitate the precipitation process. This is accomplished by utilizing a 0.45 μm polytetrafluoroethylene (PTFE) membrane that captures protein aggregates upon precipitation with cold acetone.[282] A plug in the device prevents the proteins from inadvertently passing through.[282] Upon completion of precipitation (overnight incubation at – 20 °C), the plug is removed and the SDS containing solvent is passed through the device, leaving the protein aggregates behind atop the filter.[282] The plug is once again inserted, the sample is digested, the plug is removed and the peptides are eluted through an attachable solid phase extraction (SPE) column.[282] The PTFE membrane performs separation without bias in protein or type.[282] Though MS compatible SDS levels were achieved without any additional washes, Crowell *et al*[282] recommend a single acetone wash to improve reproducibility without compromising recovery (> 95 %). The ProTrap XG is suitable for low or high protein concentrations (0.2 to 50 μg).[282] It also has the capability of being used without digestion.[282]

1.4.2.6 Commercial Options

Pierce makes SDS-Out™ precipitation kit which combines SDS precipitation with a clean-up supernatant filtration through 0.45 μm cellulose acetate membrane. This approach can partially remove SDS from protein samples that were digested in the presence of 0.1 % SDS.[155] However, as stated by Pierce, it does not remove SDS that is bound to protein. Though the SDS-Out reagent is a proprietary chemical and the chemical formula remains unknown, it is reminiscent of KCl precipitation.[274]

Pierce also makes detergent removal spin columns that contain a proprietary affinity resin that binds SDS and allows peptides to flow through. The limit of SDS removal to levels suitable for mass spectrometry analysis occurs at > 4 % SDS.[283] When starting at 2% SDS, this method consistently removes SDS to 10 ppm or below.[237] Lower SDS concentrations creates the possibility of non-specific binding of the peptides to the resin, resulting in decreased yields.[283] For peptides at 0.1 mg/mL, 2-3% SDS provided the best recovery and most efficient SDS removal.[283] Though it claims to be amenable to both protein or peptide level depletion, poor recovery is attained at the protein level.[237]

To improve the method, several alterations to the manufacturers protocol have been tried. By reducing the volume of the proprietary resin to 100 μL (1/5 of the original volume) SDS was still removed and protein recovery improved.[152] The authors state that they recover more peptides than FASP, that they trend towards being more hydrophobic (based on GRAVY[127] scores), have larger propensities to be buried into the membrane,[284] and there is greater reproducibility, but these claims are marginal at best (*e.g.* an increase to 0.54 ± 0.04 from 0.53 ± 0.04 for the propensity to be buried).[152]

Many of the other commercially available options simply make use of previously described methods. For instance, there are dialysis cassettes made by Thermo Scientific, Thermo Desalt Spin columns (size exclusion columns), and the ProteoSpin™ (ion exchange column). However, as discussed throughout Section 1.4.2, these methods all have limitations.

1.5 Research Objectives

Literature discloses many methods to remove of SDS from protein samples. The popularity of the FASP method highlights a need for a proven approach to SDS removal. However, several other studies highlight the limitations of these and other SDS removal strategies. In short, obtaining sufficient protein purity while also maintaining high recovery, in a reproducible format, remains a difficult task. As such, there is not yet that one preferred method that researchers use.

To effectively evaluate SDS depletion methods, quantitative information regarding protein purity and protein recovery is required. Most publications that evaluate the effectiveness of SDS depletion protocols do not include this data in their assessment. If this information was provided, direct critical comparisons of methods could be made. There are comparison studies of SDS depletion technologies that summarize select techniques, but they typically only discuss 2 to 3 similar depletion methods (*e.g.* precipitation).[152,220,237,276,285,286] Thus a comprehensive evaluation of published protocols is still needed.

The publications that are typically found that discuss SDS depletion report their success as a function of the number of proteins identified. Indirect comparisons such as these are also of little use as one must consider several factors that account for the differ-

ences in the number of protein identifications. These considerations include, but are not limited to, sample type, preparation techniques, and instrumentation effects.[76,287] Though there are databases that try to address this issue,[288] there are very few journal articles that provide quantitative protein recovery data, which makes comparing and contrasting SDS depletion technologies across literature futile.

An example of indirect comparisons to evaluate methods can be found with Manza *et al*[269] *vs.* Wisniewski *et al*[153], where both groups use molecular weight cut-off (MWCO) spin-filters to remove SDS prior to mass spectrometry analysis. Both methods are quite similar where the only advance of Wisniewski *et al*'s method is the addition of urea.[270,272] While defending their originality, Wisniewski and Mann state that "Manza *et al* identified 75 soluble cytosolic and 142 nuclear proteins. In contrast, our FASP approach allowed us to identify more than 7,000 proteins."[270,272] Though the addition of urea may have improved the method, claiming that it was 32x better is not appropriate considering it wasn't until five years later that, using a state of the art mass spectrometer, > 2150 HeLa proteins could be identified in a single-shot experiment.[289] Wisniewski and Mann identified 7000 proteins because they incorporated a fractionation step that divided the sample into 12 different fractions. The sum of all the proteins identified across those 12 fractions was 7000 (~ 580 proteins / sample). In addition, they were using a more modern instrument with increased resolution, scan speed, and sensitivity. Though such a comparison is obviously flawed, this sort of indirect comparison is typical in proteomics, as researchers tend to focus on the outcome of the experiment. Thus there is a need for an objective, quantitative analysis of SDS depletion technologies.

With advances in mass spectrometry technologies, the field of proteomics is growing, but front-end sample preparation needs refining.[85] As discussed in Section 1.3.1, the inclusion of SDS in proteomic workflows is important for cell lysis and protein denaturation and solubilisation. Since it is commonly used as a first step in protein workflows, samples can easily be transitioned to a protein level fractionation strategy such as GELFrEE. GELFrEE is an effective tool to use prior to top down MS analysis, which involves 4 distinct steps: (1) obtain cellular sample, (2) extract proteins from other cellular components, (3) use GELFrEE to separate proteins, and (4) analyze by LC-MS/MS.

The removal of SDS prior to LC-MS/MS is needed to avoid reduced chromatographic separation and MS signal suppression. There are numerous protocols available to deplete SDS from protein samples ahead of LC–MS, though critical evaluations of these methods is lacking. The effectiveness of these methods is best described in terms of both protein purity and protein recovery. Using a combination of assays to measure both residual SDS and protein recovery, eight of the most common SDS depletion methods are evaluated in a systematic, quantitative manner and are presented in Chapter 2. These eight methods were chosen because they provide a good representation of all types of SDS removal methods available (*i.e.* precipitation, filtration, column-based, electrophoresis) and have the potential to deplete SDS to levels compatible with MS without customized equipment.

Following the evaluation of these methods, the optimization of a novel method is presented in Chapter 3. This method, termed transmembrane electrophoresis (TME), incorporates the principles of dialysis with an applied electric current to overcome the SDS – protein binding energy needed to deplete bound SDS. It is suitable for all proteins, in-

cluding membrane proteins, and quickly and reproducibly depletes SDS to levels compatible with mass spectrometry, while retaining > 90 % protein in the sample. It is amenable to top-down MS-based protein identifications. With the development of TME complete, Chapter 4 explores improvements that have been made to the system, mainly in terms of increasing the rate of SDS depletion without compromising recovery. Parameters that were assessed include the size of MWCO membrane used, the operating temperature, and the buffers used in sample preparation. Although this list is not exhaustive of all the parameters that can play a role in improving TME, they did significantly decrease the operating time from one hour to ten minutes. With these improvements in place, Chapter 5 provides a summary of the results presented in this thesis, followed by future research objectives.

Chapter 2: Assessment of SDS Depletion Techniques [*]

2.1 Introduction

With many methods available to deplete SDS from protein samples, there exists a need for an objective quantitative comparison. Here, a standardized system is employed to test the effectiveness of SDS depletion, considering both the recovery and purity of protein and peptide samples. To evaluate protein recovery and protein purity of these methods, reliable assays are needed. For protein purity, colorimetric methods such as MBAS (methylene blue active substance) and Stains-All are employed. But like all colorimetric methods, they can be susceptible to interferences. Another method, developed previously in our lab, utilizes LC-MS to accurately and precisely measure residual SDS. In terms of protein recovery, BCA (Bicinchoninic acid assay) and LC-UV protocols were utilized.

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Solutions containing low and high concentrations of BSA, or of an *E. coli* proteome extract were evaluated to test the effectiveness of eight independent SDS depletion protocols. Differences in the efficiency of the methods were revealed, most notably with respect to the recovery of protein or peptide following SDS depletion. The impact of protein recovery and purity resulting from two SDS clean-up approaches (FASP and acetone precipitation) on their capacity to identify proteins by LC–MS were further assessed.

2.2 Experimental

2.2.1 Chemicals and Reagents

Bovine serum albumin (BSA), and TPCK-treated trypsin (T8802) were purchased from Sigma (Oakville, Canada). Milli-Q water was purified to 18.2 MΩ cm. Organic solvents (acetone, methanol, chloroform, acetonitrile, isopropanol) were of HPLC grade and obtained from Thermo Fisher Scientific (Ottawa, Canada). Methylene blue was also from Fisher. Reagents for casting and staining SDS PAGE gels, as well as urea, dithiothreitol (DTT), iodoacetamide (IAA), and SDS were from Bio-Rad (Mississauga, Canada). Formic acid (98%) was from Fluka (Mississauga, Canada), while trichloroacetic acid (TCA), trifluoroacetic acid (TFA), Stains-All, and all remaining chemicals used were from Sigma.

2.2.2 Bovine Serum Albumin (BSA) Preparation

Stock solutions of 2.0 g/L BSA were prepared in Milli-Q water as determined by the molar absorbtivity, 279 nm, (ϵ) 43,824 $M^{-1}cm^{-1}$.[290] The solution was dispensed into 500 µL aliquots and stored at -20 ºC until ready for use.

2.2.3 *E. coli* Growth and Protein Extraction

E. coli was cultured according to established protocols.[291] Cells were grown in liquid broth (LB) media at 37 ºC with shaking until an OD_{600} of 0.7, then isolated by centrifugation at 5000 × *g* (15 min). Proteins were extracted by suspending the cells in 2 % SDS with heating (95 ºC for 5 min). Cellular debris was pelleted by centrifugation (15,000 × *g*, 15 min) and discarded. The total protein content of the supernatant was determined using a BCA assay kit from Pierce (Rockford, IL), against a calibration curve of BSA in 2 % SDS.

2.2.4 Sample Preparation for SDS Depletion

Stock solutions of BSA and of the extracted *E. coli* proteins were each prepared at concentrations of 0.1 g/L and of 1.0 g/L with1% SDS in water. Five 100 µL aliquots were prepared as replicates for each of the eight SDS-depletion protocols. For peptide level SDS depletion, the 100 µL protein aliquots were diluted to a final concentration of 0.025% SDS and digested with trypsin as described by Sun *et al.*[261] Following digestion and acidification, samples were evaporated to dryness in a Speedvac, and reconstituted with sonication in 100 µL of the appropriate buffer system, restoring the SDS concentration to 1%.

2.2.5 SDS Depletion Methods

2.2.5.1 FASP

As described by Wisniewski *et al*[153,271] a 30 kDa Micron YM-30 filter (Millipore, Cat. No. 42409) was selected, employing 8 M urea in 0.1 M Tris (pH 8.5) to facilitate removal of protein-bound SDS. 10 kDa YM-10 filters (Millipore, Cat. No. 42406) were also assessed, as were Amicon Ultra 2 10 kDa filters (Millipore, Cat UFC201024). In brief, the sample (100 µL) was mixed with 200 µL of urea buffer A (UA) (8 M Urea in 0.1 M Tris pH 8.5) in the filter unit and centrifuged at 14,000 × *g* for 15 minutes. An additional 200 µL of UA was added to the filter unit and centrifuged at 14,000 × *g* for 15 minutes. The flow-through was discarded from the collection tube. To the filter unit, 100 µL of IAA solution (0.05 M IAA in UA) was added and mixed for one minute, then incubated without mixing for 20 minutes. The filter units were centrifuged at 14,000 × *g* for 10 minutes. To the filter unit, 100 µL of UA was added and centrifuged at 14,000 × *g* for 15 minutes. This step was done twice. Following that, 100 µL of ammonium bicarbonate (ABC) (0.05 M) was added to the filter unit and centrifuged at 14,000 × *g* for 10 minutes. This step was done twice. Following that, 40 µL of ABC with trypsin (enzyme to protein ratio 1:100) was added and mixed for one minute. The cleaned proteins were digested overnight, incubated in a wet chamber at 37 °C. The resulting peptides were released from the filter by centrifugation (14,000 × *g*, 40 min), followed by a wash of the filter in 0.5 M NaCl. The collected sample was acidified with CF_3COOH and the peptides were desalted as described by Wisniewski *et al*[153] using 10 mg Oasis HLB sample extraction columns (Waters, Milford), eluted with 1.25 mL of 70% ACN, dried and diluted to a final volume of 100 µL with water, with sonication.

2.2.5.2 Protein Precipitation with 80 % Acetone

As described by Botelho *et al*,[164] proteins were precipitated through addition of 400 µL acetone, with overnight incubation at -20 ºC and isolation of the pellet through centrifugation (15 min, 21,000 × *g*). The majority of the supernatant was removed by micropipette; leaving 10 – 50 µL behind while ensuring the pellet was not disturbed. The protein pellet was subject to two additional washing steps with 400 µL of acetone and the supernatant was again removed. Any remaining solvent was left to evaporate and the final dry protein pellet was suspended in 100 µL water, with sonication. Sixty µL were reserved for the SDS assays. The remaining 40 µL of sample was diluted with 40 µL of 2 % SDS, sonicated to fully dissolve the suspended protein, and subjected to BCA protein assay.

2.2.5.3 Protein Precipitation in TCA with Acetone Wash

Proteins were precipitated through addition of 1/10th volume of 100 % (w/v) TCA.[292] The protein pellet was collected by centrifugation (15 min, 21,000 × *g*) and incubated overnight in 1 mL cold (-20 ºC) acetone. The protein pellet was again collected by centrifugation, and the remaining solvent was left to evaporate. The final dry protein pellet was dissolved in water.

2.2.5.4 Surfactant Precipitation with 0.5 M KCl

As described by Zhou *et al*,[274] 50 µL of the protein solution was combined with an equal volume of 0.5 M KCl, with incubation at room temperature for 5 min. The samples were centrifuged (14,000 × *g*, 10 min) to pellet the detergent and the supernatant (100 µL) was retained for analysis of protein recovery.

2.2.5.5 Pierce Detergent Removal Spin Columns

The spin cartridges were obtained from Pierce (Rockford, IL) and contain a proprietary affinity cartridge that binds SDS allowing purified proteins as well as peptides to flow through. The cartridges were used according to the manufacturer's recommended protocols, and as described previously.[283] Prior to loading the intact proteins or the digested peptides, the storage solution of the spin cartridges (0.5 mL column) was removed by centrifuging at 1500 × *g* for one minute. The spin columns were then equilibrated with 2 x 400 µL of 50 mM Tris buffer (pH 8), with removal by centrifuging at 1500 × *g* for one minute. Samples (100 µL) were added directly atop the resin. Following a two-minute incubation, the flow through containing purified protein or peptides was collected in new collection tubes by centrifuging the spin columns at 1500 × *g* for two minutes.

2.2.5.6 Strong Cation Exchange

A HPLC approach for peptide level SDS depletion was performed as described by Sun *et al*,[261] with minor modification to the final solubilization. Samples (100 µL) dissolved in 50 mM NH_4HCO_3 were reduced with 6 µL of 900 mM DTT at 37 ºC for one hour. Alkylation was then done with 60 µL of 200 mM IAA at room temperature in the dark for one hour. Tryptic digestion was then done at a mass ratio of 1:50 for 48 hours at 37 ºC. The samples were then dried down in a SpeedVac, then heated for two hours at 85 ºC to remove ammonium bicarbonate. After reconstituting the samples in 1% SDS with phosphoric acid (pH 1 – 2), the SDS was removed using a self-packed 1.0 × 100 mm polySULFOETHYL A SCX column (PolyLC, Columbia, MD), with mobile phase A as 10 mM KH_2PO_4 at pH 2.67 (adjusted with phosphoric acid), and mobile phase B as 500 mM KCl and 10 mM KH_2PO_4 (pH 2.67), at a flow rate of 40 µL/min. Following a 10

min flush in mobile phase A, peptides were eluted and collected as a single fraction by switching to 100% B. The salt was removed from the collected fractions as described in Section 2.2.7.3.1, with final reconstitution in 100 µL of water prior to the SDS and peptide assay.

2.2.5.7 SDS PAGE and In-Gel Digestion

SDS-PAGE used gels cast to 15 % T (T = total acrylamide). The gels containing BSA were resolved at 200 V for 60 min, while gels containing *E. coli* proteins were resolved at 200 V for only 15 min, being just sufficient to allow all protein to enter the resolving gel. Following coomassie staining, BSA was dissected as a 2 mm wide band, while *E. coli* proteins were excised from the gel as a single 1 cm wide band. In-gel tryptic digestion was performed as described by Shevchenko *et al*.[236] Briefly, the protein containing bands were placed in microcentrifuge tubes and incubated for 10 minutes in 500 µL of ACN. The pieces were spun down and all supernatant was removed. They were then reduced using enough 10 mM DTT to cover the gel pieces and incubated at 56ºC for 30 minutes. The samples were cooled and incubated for 10 minutes with 500 µL of ACN, before being spun down and removing the supernatant. The samples were then alkylated with enough 55 mM IAA to cover the gel pieces and incubated for 20 minutes in the dark. The samples were once again incubated for 10 minutes with 500 µL of ACN, before being spun down and removing the supernatant. The samples were then digested using enough 13 ng/µL Trypsin to cover the gel pieces, incubated at 4ºC for 30 minutes, topped up with additional Trypsin, and incubated at 4ºC for an additional 90 minutes. Using 100 mM ABC, enough solution was added to the tubes to ensure that the gel pieces would be covered while being incubated overnight at 37ºC. The following

day, peptides were extracted with 2 × 200 µL aliquots of 2:1 acetonitrile: 5% formic acid in water. The combined extracts were evaporated to dryness in a Speedvac. Peptides were reconstituted in 100 µL water, with sonication for 10 minutes.

2.2.6 SDS Assays

2.2.6.1 LC-MS/MS

Previously developed by Kegan Stephen from the Doucette group, samples were subject to a minimum of three replicate injections of 5 µL, loaded onto a self-packed 1 × 135 mm column containing 10 µm C18 beads (Grace Vydac) through an Agilent 1050 series autosampler. The column temperature was maintained at 40 ºC, and the flow rate was 50 µL/min. An isocratic solvent system comprising 69.9 % acetonitrile, 0. 1% formic acid in water was used to elute the SDS from the column, directing the eluent to a Waters Quattro LC Micromass Triple Quad MS (Milford, MA) with an electrospray ionization source (ESI) source operating in negative mode. A capillary voltage of 3.50 kV was used, and collision energy of 39 with argon collision gas. SDS was observed through the transition from *m/z* 265 → 97. Peak areas were extracted using the MassLynx software, associated with the MS instrument.

2.2.6.2 Methylene Blue Active Substance (MBAS)

The methylene blue active substances assay (MBAS) was performed as described by Arand *et al*.[293] In brief, 100 µL of appropriately diluted sample was combined with 100 µL of methylene blue reagent (250 mg methylene blue, 50 g sodium sulfate, 10 mL sulfuric acid diluted to 1.0 L), vortexed briefly, then mixed with 400 µL chloroform. The samples were vortexed and centrifuged (1500 × *g*), retaining the lower chloroform layer

for absorbance measurement at 652 nm on an Agilent 8453 spectrophotometer (Mississauga, Canada).

2.2.6.3 Stains-All

As described by Rusconi *et al*,[294] appropriate volumes of the sample were added to 200 µL of the intermediate Stains-All solution until a yellow color change was observed. Samples were assayed within 15 min and the absorbance was measured at 453 nm against an SDS calibration curve prepared alongside each data set.

2.2.7 Protein / Peptide Recovery Analysis

2.2.7.1 Bicinchoninic Acid (BCA) Assay

Intact protein recovery was determined through a Pierce™ BCA protein assay. Samples were diluted in 1 % SDS, to which 15 µL was combined with 300 µL of BCA working reagent. Samples were heated in a 57 ºC water bath for 30 min, then cooled to room temperature prior to recording the absorbance at 562 nm. BSA was used to construct a calibration curve for all test samples, and was prepared in 1 % SDS.

2.2.7.2 LC/UV Assay

Approximately 5 - 10 µg of sample were injected onto a self-packed 1 × 100 mm C18 column (Waters ODS 5 µm beads) using an Agilent 1200 high performance liquid chromatography (HPLC) series instrument. After a five minute hold, peptides were eluted as a single fraction through an instantaneous ramp from 5 % to 80 % acetonitrile, with the resulting UV absorbance signal monitored at 214 nm against a calibration curve consisting of BSA.[295]

2.2.7.3 Peptide Desalting

Approximately 5 μg of sample were injected onto a self-packed 1 × 100 mm C18 column (Waters ODS 5 μm beads) using an Agilent 1200 high performance liquid chromatography (HPLC) series instrument with UV detection at 214 nm. After a five minute hold, peptides were eluted as a single fraction through an instantaneous ramp from 5 % to 80 % acetonitrile. The sample was collected while an UV absorbance signal was monitored at 214 nm.

2.2.7.3.1 Peptide Recovery from Desalting

Three 100 μL aliquots of 0.1 g/L digested BSA peptides were subjected to the LC/UV desalting procedure and the peptide fraction was collected. Two of those samples were dried down in a Speedvac and reconstituted in water, or water with 5 % ACN, and sonicated for 10 min. The third fraction acted as the control, reduced the volume to the original 100 μL to allow reinjection without excessive organic solvent. The three samples were then analyzed by LC/UV using the assay described in Section 2.2.7.2.

2.2.8 GELFrEE

As described by Botelho *et al*,[164] 100 μg of *E. coli* proteome extract was loaded onto each of four 6 mm i.d., 12 % T gel columns. A voltage of 250 V was applied and a total of 15 fractions were collected, according to the following time scheme: dye front (fraction 0), 5 × 1 min, 5 × 2 min, 3 × 5 min, 1 × 15 min. Fractions from a given collection interval were pooled across the four columns. The combined fractions were visualized by SDS-PAGE with coomassie staining. Fractions 4 and 5 (MW 30–45 kDa) and

fractions 12 + 13 (55–80 kDa) were then combined to form the low mass and high mass fractions, respectively (total volume of each fraction ~800 μL).

2.2.9 MS of GELFrEE fractions

One hundred microliters each of the high mass and low mass fractions were individually subject to acetone or FASP II for SDS depletion. Following acetone precipitation, the resulting protein pellet was resolubilized in 8 M urea, then diluted and digested with trypsin (100 ng) as described previously by Botelho *et al*.[164] With FASP II, 10 kDa Micron YM-10 (Millipore) were used. The digested samples were desalted and peptides were quantified by LC/UV as described in Section 2.2.7.2.

Peptides were analyzed on an Orbitrap Velos Pro (Thermo Fisher Scientific) coupled to a Dionex Ultimate 3000 Rapid Separation LC nanosystem (Bannockburn, IL), operating in MS mode at a resolution of 30,000 FWHM, scanning in rapid mode for MS/MS (66,666 Da/s, at <0.6 Da FWHM). The column was a self-packed monolithic C18 (0.1 × 150 mm, Torrance, CA), coupled to a 10 μm New Objective PicoTip non-coated Emitter Tip (Woburn, MA). The gradient from solvent A to solvent B was as follows: 0 min, 3 % B; 3 min, 3 % B; 5 min, 5 % B; 69 min, 35 % B; 72 min, 95 % B; 77 min, 95 % B; 80 min, 3 % B. The Orbitrap was set to data dependent mode (MS followed by MS/MS of the top 10 peaks with minimal signal threshold of 3×10^4 and a charge state between +2 and +4). Dynamic exclusion was applied for 25 s over a range of ± 5 ppm.

2.2.10 Data Analysis

Peptides were identified using the Proteome Discoverer software, searching the *E. coli* database (downloaded May 2014, 4269 entries), and allowing modifications of oxidized methionine or carbamidomethylation at cysteine. The mass tolerance was set to 10 ppm (MS mode) and 0.8 Da (MS/MS mode), with 2 missed cleavages assigning a peptide false positive rate of 1%, and minimum 2 peptides per protein. Cellular component was determined using Gene Ontology functional annotation from DAVID (Database for Annotation, Visualization, and Integrated Discovery). Hydrophobicity was determined from GRAVY scores obtained from www. bioinformatics.org/sms2/protein gravy.html.

2.3 Results and Discussion

Numerous methods are available for SDS depletion. Table 2-1 lists the methods evaluated, encompassing techniques applicable to protein and peptide level SDS depletion. Beginning in 1% SDS, the four test solutions comprise either a single protein (BSA) or a proteome mixture, prepared at two concentrations (0.1 and 1.0 g/L). These test solutions provide a standardized means of assessing the efficiency of the depletion technique, as we quantify the residual SDS together with protein or peptide recovery.

Table 2-1. SDS depletion protocols evaluated.

	SDS Depletion Method	Reference	Form Of:	
			Initial Sample	Final Sample
1	In-gel digestion following SDS PAGE	236	Protein	Peptide
2	FASP	153,271	Protein	Peptide
3	Protein precipitation in 80 % Acetone	164	Protein	Protein
4	Protein precipitation in TCA	[292]	Protein	Protein
5	Detergent precipitation in 0.5 M KCl	274	Protein	Protein
6	Pierce detergent removal spin cartridge	283	Protein	Protein
7	Pierce detergent removal spin cartridge	283	Peptide	Peptide
8	Strong cation exchange	261	Peptide	Peptide

Owing to its high sensitivity and throughput, the MBAS assay is a convenient approach to quantify residual SDS. Because, anionic compounds can ion pair with the cationic methylene blue dye, this method is prone to interferences. As an example, employing the FASP protocol, the MBAS assay revealed an apparent level of SDS depletion between 45 and 60 % (data not shown). Such values are suspiciously poor, given that over 99 % SDS must be depleted for samples to be compatible with LC–MS. The FASP protocol was repeated, omitting the addition of TFA (used to quench trypsin). From the test solutions, MBAS reported an average 99.6 % of the SDS to be depleted. This value would appear more reasonable, though it may still be possible that other interfering compounds skew the analysis. Given the non-selective nature of a colorimetric assay, we sought to develop a more reliable assay to quantify trace levels of SDS from protein containing solutions.

Table 2-2. Analytical figures of merit for SDS quantitation.

SDS Assay	LOQ[a] (ppm)	LOQ (μg)	Linear Range (ppm)	R^2	Interday Repeatability[b] (% RSD)
LC-MS/MS	0.9	0.004	1-50	0.998	4
MBAS	2	0.4	2-14	0.996	9
Stains-All	1	0.1	5-50	0.968	14

[a] Limit of quantitation (LOQ) is calculated at S/N = 10.
[b] Repeatability determined from slope of calibration curve (n=6).

A high throughput LC–MS/MS approach was previously developed to detect SDS.[296] The method employs a rapid isocratic separation of SDS at elevated temperature to isolate the surfactant from interfering compounds (salts, buffer components), allowing quantitation in a fully automated fashion. The final operating conditions selected for the

LC–MS/MS assay was a column temperature of 40 ºC and an isocratic separation in 70 % acetonitrile. Under these conditions, a diversion valve directs the first three min of solvent to waste, minimizing contamination of the ESI source. The SDS peak has a retention time of 4.4 min while the total run time is 15 min. Table 2-2 summarizes the figures of merit attainable with the LC–MS/MS approach. For comparison, the figures of merit for SDS colorimetric assays (MBAS and Stains-All) were also determined. Of the three techniques, LC–MS/MS offers the widest linear dynamic range (Figure 2-1), with an R^2 of 0.998 obtained between 1 and 50 ppm SDS. The approach also provides the lowest limit of quantitation, 0.9 ppm from a 5 µL injection which corresponds to below 5 ng SDS. The concentration limit is similar to that of Stains-All (1 ppm) though the colorimetric assay employed a sample volume of 100 µL, corresponding to 0.1 µg SDS. As seen in Table 2-2, LC–MS/MS also provides the highest day-to-day repeatability (4% RSD). No deterioration in MS signal intensity was observed over several weeks of data collection. This is attributed to the small amount of SDS injected (5 µL, generally containing under 10 ppm SDS). The influence of salt, TFA, and inclusion of protein in the calibration solutions to monitor the *m/z* 265 → 97 transition for SDS were also assessed. As shown in Figure 2-2, incorporation of 100 mM NaCl in the sample causes a 30 % decrease in the SDS signal; a similar drop in signal was observed when 0.5 g/L BSA was included in the sample. However, given the low limit of quantitation, these potential matrix effects can be alleviated by diluting the sample prior to injection.

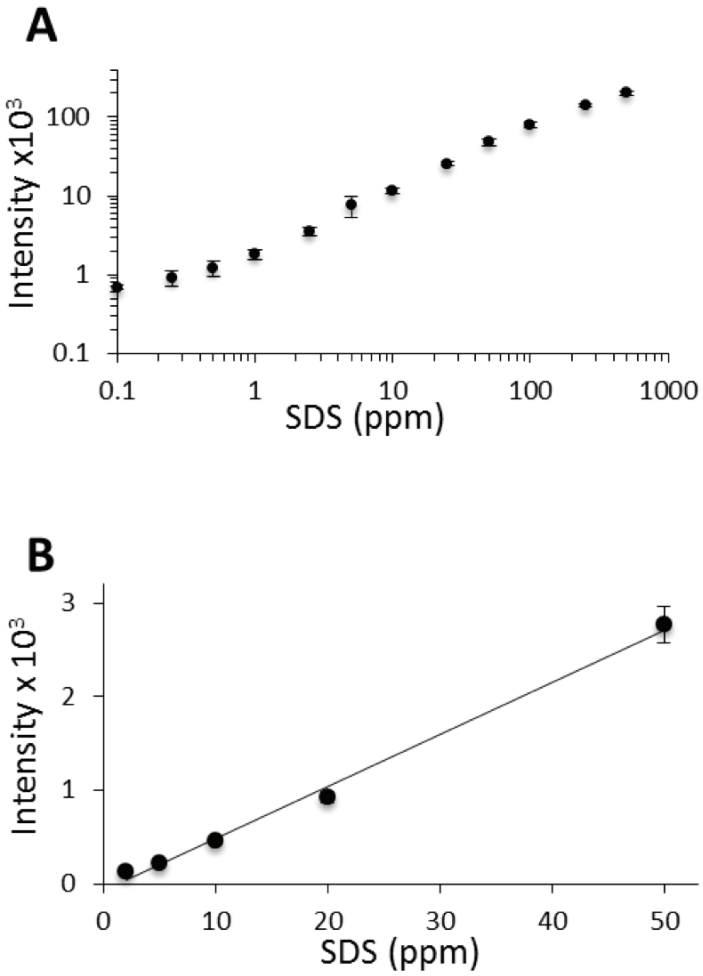

Figure 2-1. (A) The LC-MS/MS assay showing quantitation of SDS over a wide range. The amount of SDS (ppm) is plotted against the peak area in a log vs log plot. In **(B),** an expanded view is shown, relating to the linear portion of the curve. Error bars represent standard deviation from replicate injections (n=2).

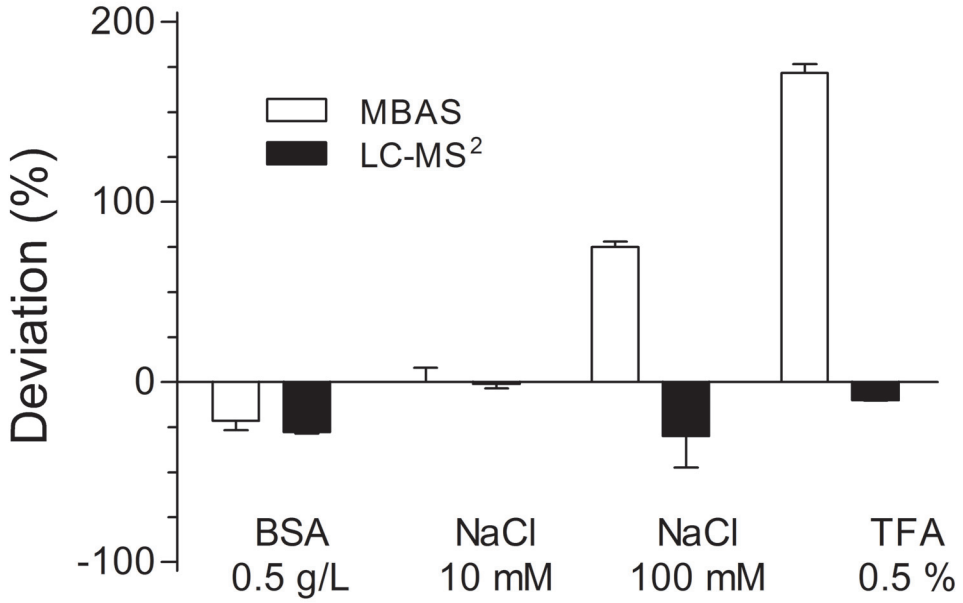

Figure 2-2. The influence of sample additives on quantifying 0.001% SDS (10 ppm) through LC-MS/MS or through a MBAS colorimetric assay. The figure depicts the % deviation in the reported concentration of SDS relative to a control solution of 10 ppm SDS prepared in the absence of additives. Error bars depict the standard deviations from replicate analysis of three independent solutions. * MBAS data previously obtained by Kegan Stephen and LC-MS/MS data obtained by Carolyn Kachuk.

Figure 2-3 summarizes the quantity of residual SDS remaining from each of the eight depletion protocols, as reported with the LC–MS/MS assay. The residual SDS from each protocol by MBAS as well as the Stains-All approach was also assayed (Figure 2-4). While the three assays show general agreement, there were noted exceptions, as seen with the FASP approach discussed above. Differences were also seen from the SDS PAGE digestion protocol, which yielded negative deviations in the colorimetric assays relative to LC–MS/MS (Figure 2-4). Given the improved selectivity of LC–MS/MS over the colorimetric assays, emphasis is placed on the assessment of SDS by mass spectrometry. Beginning with 1% SDS and assuming a constant sample volume of 100 µL follow-

ing SDS depletion, the cut-off limit for MS analysis of a protein sample necessitates that greater than 99 % of SDS be depleted (*i.e.* under 10 µg of residual detergent). As seen in Figure 2-3, the efficiency of SDS depletion varies considerably across methods. The least efficient approaches for SDS depletion involve precipitation of the surfactant with 0.5 M KCl, as well as protein precipitation with TCA. Using these methods, residual SDS varied considerably depending on the test solution used (low or high protein concentration, single protein or proteome mixture).

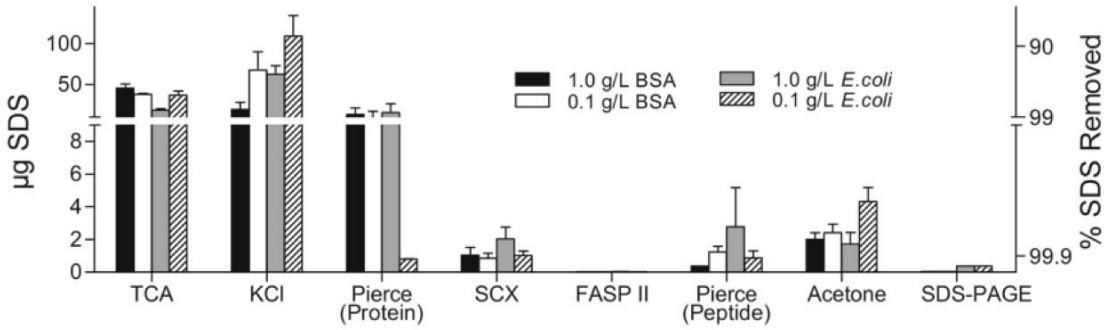

Figure 2-3. Summary of residual SDS as determined by the LC-MS/MS assay following detergent removal through one of eight protein purification protocols (* refers to levels below limit of detection). The four test solutions initially contained 1 % SDS, with an initial volume of 100 µL (initial SDS = 1000 µg). Error bars represent the standard deviation of five independent samples being subject to the SDS depletion protocol.

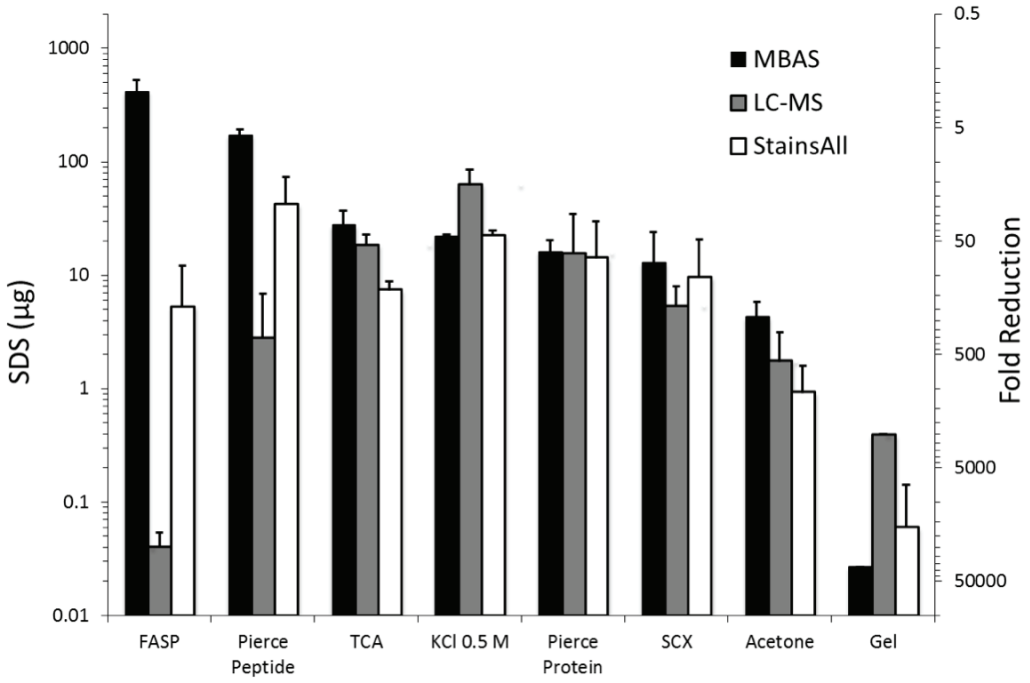

Figure 2-4. For the eight detergent depletion protocols, residual SDS was quantified using each of three assays. Methylene Blue Active Substances assay (MBAS), the commercial Stains-All colorimetric assay, and the LC-MS/MS assay described here. The test solution was the 1 g/L *E. coli* proteome mixture, initially containing 1 % SDS and corresponding to 1000 µg detergent. Error bars depict standard deviations (n=3).

However, for all test solutions using KCl and TCA precipitation, SDS depletion did not reach the 99 % threshold. As previously reported, KCl will precipitate free SDS, but not that which is bound to protein.[297] Given the levels of residual SDS attained here, it is evident that at least some protein bound SDS has been removed. One would also expect a higher level of residual SDS from the more concentrated protein samples, which was observed, though only a ~2–3 fold increase in SDS from a 10-fold increase in protein. Zhou *et al.*[274] reported 99.9 % SDS to be removed by KCl; their protocol was applied at the peptide level and so binding to SDS would not be as strong. This level of depletion was also not quantified directly, but rather by extrapolation from visualizing the

potassium dodecyl sulfate (KDS) pellet at increasingly lower concentrations of SDS. Considering TCA precipitation, the higher residual surfactant was attributed to the co-precipitation of SDS bound to protein.[298] While a subsequent acetone wash aids in removing SDS, this is evidently not as effective as other purification protocols. It should be noted that LC–MS/MS analysis of a purified sample may still be possible following KCl or TCA depletion; the 0.01 % SDS threshold described here is based on previous work by our group assessing the analysis of a 10 μL solution containing 1 μg digested peptides, and employing specific LC–MS operating parameters.[164] Higher concentrations of protein may tolerate higher levels of SDS. Nonetheless, it is clear that other depletion strategies exist which are more effective at SDS removal.

The Pierce detergent removal cartridge yielded notable differences between protein level and peptide level SDS depletion. As stated by the manufacturer, the cartridge removes over 95 % of detergents. From Figure 2-3, when applied to intact proteins, SDS depletion approached the 99 % removal threshold – well above the manufacturer specifications, though still less than other depletion protocols. A lower level of residual SDS observed for the 0.1 g/L *E. coli* protein test solution may be attributed to the high variance in the depletion technique (~100 % RSD). By contrast, peptide level depletion with the Pierce cartridge consistently yielded lower levels of residual SDS. Such results are to be expected as protein–SDS interactions are weaker at the peptide level. The level of SDS remaining from the Pierce cartridge is similar to that of SCX depletion, which also employs a peptide level clean-up. Of the methods tested, acetone precipitation was the only protein level approach that consistently depletes over 99 % of the SDS.

Of the eight methods assessed, the highest detergent removal efficiency was observed with FASP (30 kDa membranes). From Figure 2-3, with all four test solutions, FASP consistently depleted SDS to levels below the limit of quantitation of our LC–MS/MS assay. This corresponds to well over 99.99% SDS depletion. Using 10 kDa filters, the residual SDS was slightly higher (between 3 and 4 μg), though still among the better performing SDS depletion protocols, and well below the 10 μg cut-off permitting LC–MS analysis. Wisniewski has previously shown that larger MWCO filters are more efficient at SDS depletion.[271] An exceptional level of SDS depletion was also observed through in-gel digestion of the BSA test samples, though detectable levels of SDS were observed with the *E. coli* test samples. With *E. coli*, a larger (~1 cm) gel band was excised to recover the full *E. coli* proteome from the gel. The shorter resolving period, which may not be sufficient to isolate the higher concentration of SDS from the sample loading buffer is also noted. It can therefore be stated that FASP is as effective as a conventional in-gel digestion protocol in terms of SDS depletion.

Though certain depletion methods are favoured in terms of protein purity, one must also consider protein recovery in assessing the efficiency of the separation protocol. Figure 2-5 summarizes the recovery of protein or peptide obtained though the various SDS depletion methods. Table 2-3 compares the recovery values obtained here to those previously reported in the literature. As seen for the majority of these methods, the recovery varies considerably and depends on sample composition and concentration. TCA precipitation was completely ineffective at recovering the BSA sample. This protein could be precipitated in high yield when SDS was omitted from the solution, indicating that the

surfactant has a controlling influence on the recovery of protein through TCA precipitation. Focusing on the TCA recovery values obtained for the *E. coli* mixture prepared in 1 % SDS, our values are consistent with those previously reported.[277] Other single protein mixtures may be more amenable to TCA precipitation, though it is realized that the ideal purification strategy would be applicable to all sample types.

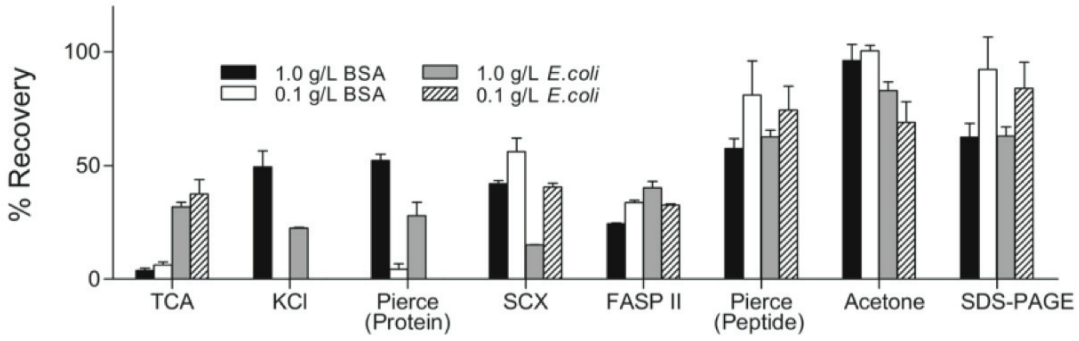

Figure 2-5. The recovery of protein or of peptide following SDS depletion as determined by BCA or LC-UV assay, respectively (* refers to levels below limit of detection). The four test solutions were at an initial volume of 100 µL (initial SDS = 1 %). Error bars represent the standard deviation of five independent samples being subject to the SDS depletion protocol.

Table 2-3. Comparative assessment of protein recoveries to literature values.

	SDS depletion method	Protein recovery (%)		
		Current Study	Literature	Reference
1	In-gel digestion following SDS PAGE	62-92	20-90	[144,299]
2	FASP	24-40	10-100	[12,153,273,299]
3	Protein precipitation in 80% Acetone	68-110	50-104	[276,277,298,300–302]
4	Protein precipitation in TCA	5-37	25	[298]
5	Detergent precipitation in 0.5M KCl	<LOQ-50	44-94	[236,292]
6	Pierce detergent removal spin cartridge (protein)	<LOQ-55	32-72	274
7	Pierce detergent removal spin cartridge (peptide)	63-81	20, 33	[274,292]
8	Strong cation exchange	15-56	40-90	261

KCl as well as the Pierce columns showed reduced recovery at the lower starting concentration. In 0.5 M KCl, sample loss is more severe at lower protein concentration. As seen in Figure 2-5, the 0.1 g/L test solutions yield below 5 % recovery (corresponding to the limit of quantitation for the BCA assay). Similarly, the Pierce cartridges also yield below 5 % recovery for the lower concentration test solutions. A 0.1 g/L sample would fall below the manufacturer's recommended level for use with the Pierce cartridge. Interestingly, those methods with the poorest SDS depletion efficiency (KCl, TCA, Pierce detergent removal from intact proteins), also demonstrated the lowest protein recovery. These methods all incorporate protein-level purification, which is generally more susceptible to sample loss owing to the varied solubility of intact proteins relative to peptides.

The remaining SDS depletion techniques tested continue to display varying degrees of sample loss. The highest average recovery obtained for a protein level purification was through acetone precipitation. This is consistent with our previous assessment of protein recovery in acetone where yields between 80 and 100 % are observed.[12,300] Crowell *et al* showed previously that the ionic strength of the solution was an important factor influencing the recovery of proteins.[300] Here, SDS acts as the ionic strength buffer, ensuring high yields through precipitation. The highest recovery for samples involving peptide level clean-up was obtained through in-gel digestion, wherein 10 μg total protein loaded in the gel provided greater than 90 % peptide recovery (Figure 2-5). At the peptide level, the Pierce cartridge also demonstrated higher recovery at the lower protein concentration. SCX recovery did not appear to correlate with concentration, though yields were lower than those reported previously.[261] The SCX removal protocol is adopted from Sun *et al*, wherein it was originally reported that a peptide recovery of 84 % is possible for

samples prepared in 1 % SDS.[261] Our test solutions contain considerably less protein than the previously reported study (100 or 10 µg, *vs* 3000 µg as used by Sun *et al*). Thus, given the influence of sample composition, the numbers shown here should be compared to those reported previously with caution.

Considering all four test solutions, FASP recovery varied between 24 and 40 %, with the lowest value obtained for the 1 g/L BSA sample. This range is lower than that reported by Wisniewski (~50 %),[271] though recoveries between 10 and nearly 100 % have been reported.[273] Variation in recovery has been attributed to the efficiency of tryptic digestion on the MWCO filter, particularly if any residual SDS is present.[273] The FASP protocol has also yielded variable success across different labs,[62,303] noting that some proteins display much stronger interactions with the membrane, further reducing protein yields.[304] While some discrepancies are to be expected, the low recovery observed through the FASP protocol warrants further investigation.

The recovery values reported in this study incorporated a peptide desalting step, in cases where it was integral to the depletion protocol (SCX and FASP in particular). From assessment of recovery through digested BSA, the recovery of SPE-based desalting step was determined to be approximately 90 %, thus adding to the overall loss of sample. Likewise, simply solubilizing a dried peptide fraction can attribute up to 20 % sample loss. This loss may be biased toward hydrophobic peptides; the nature of peptide loss was not assessed here. Nonetheless, it is realized that every step of the detergent depletion process has the potential to reduce recovery. However, our assessments demonstrate that the bulk of sample loss occurs prior to the desalting steps.

While FASP maximizes purity, with a near complete removal of SDS, it suffers a higher degree of protein loss relative to other approaches. Protein losses are minimized with acetone precipitation, and residual SDS still falls below the concentration that should impact LC–MS analysis of proteins. The impact of purity *vs* protein recovery on conducting bottom up proteome analysis was next assessed. In this case, a complete workup is performed for both FASP and acetone precipitated proteins, including tryptic digestion and peptide desalting. GELFrEE was used to fractionate a soluble proteome extract of *E. coli* according to molecular weight. The resulting fractions contain approximately 0.1 % SDS, at an estimated protein concentration of 0.1 g/L. Figure 2-6 depicts the resulting GELFrEE fractions, as visualized through SDS PAGE, marking the low and high molecular weight fractions selected for subsequent processing. These fractions were divided such that an equal portion could be subjected to SDS removal through either FASP or acetone precipitation. Given the presence of low MW proteins in the low MW GELFrEE fraction, a 10 kDa filter was employed for FASP in order to ensure that these proteins were recovered. Assessment of protein recovery using the *E. coli* and BSA test mixtures determined yields similar to that of the 30 kDa membrane. With 0.1 g/L *E. coli*, and in the absence of the peptide desalting step, protein recovery with the 10 kDa Micron filter was found to be 57.4 ± 0.9 %. Similarly, 52 ± 9 % yield was found through the 0.1 g/L BSA test sample using Amicon 10 kDa filters. Recovery will be somewhat lower following desalting, but is similar (if not marginally better) to that observed with the 30 kDa filters. The complete list of identified peptides and proteins from MS analysis of these fractions is attached as supplemental Files S1–S8 (Appendix A).

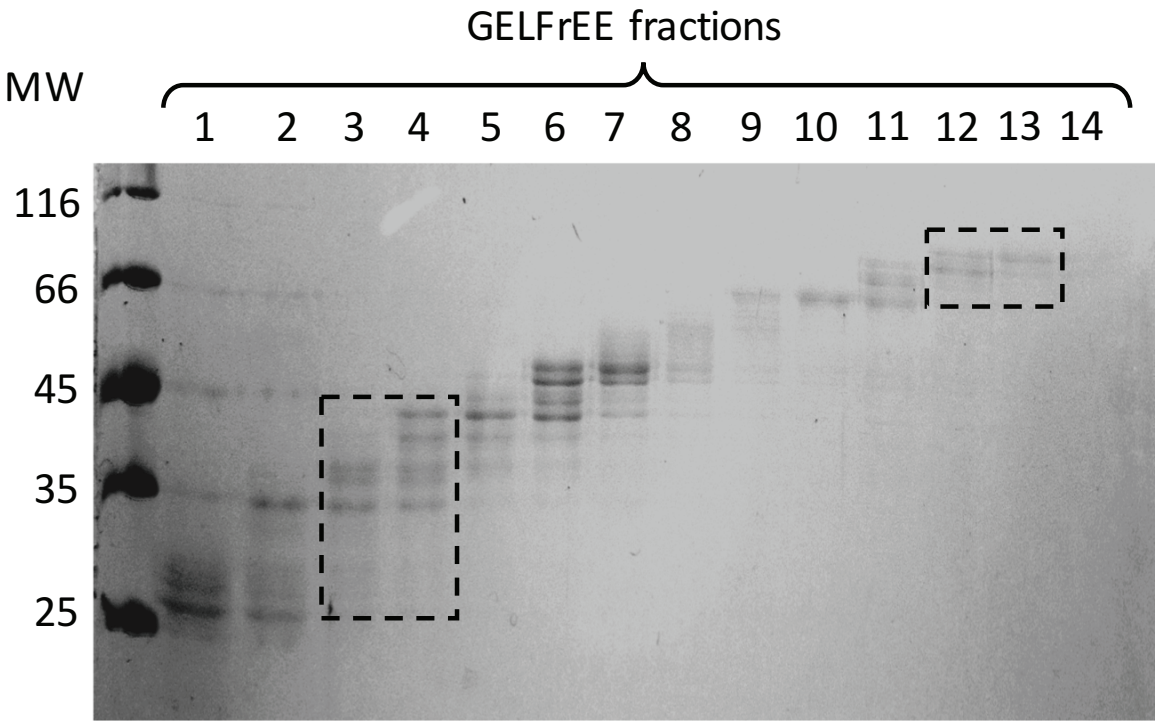

Figure 2-6. GELFrEE fractions of an *E.coli* proteome extract, as visualized by SDS PAGE with coomassie stain. Fractions 3 and 4 were combined, as were fractions 12 and 13, to form the low MW and high MW fractions which were then subjected to acetone precipitation or FASP II ahead of bottom up MS analysis of the resulting peptides.

Table 2-4 summarizes the MS identifications and reveals that, for both the low MW and high MW fractions, SDS removal through acetone precipitation results in a greater number of detections *vs.* the FASP approach. For the high MW fraction, acetone precipitation yields 13 % more proteins, and 20 % more peptide hits compared to FASP. Similarly, at the low MW fraction, acetone reveals 17 % more peptides, corresponding to a 12 % increase in the number of identified proteins. Figure 2-7 describes the total proteins identified from the two GELFrEE fractions as a function of their molecular weight, with similar distributions observed between acetone and FASP. Our group has previously shown 80 % acetone to be effective at precipitating proteins across a broad mass range,

including low molecular weight proteins.[164] Profiling the protein cellular components through Gene Ontology (Figure 2-8) also yields no discernible difference in the distribution of proteins detected following acetone precipitation or FASP, indicating that there appears to be no specific bias in the type of proteins lost to FASP or acetone. In addition, no noticeable difference in the hydrophobicity, nor isoelectric point of the resulting peptides identified from each of the two SDS depletion strategies (Figure 2-9). Residual SDS following acetone precipitation is similar to that of FASP II using a 10 kDa membrane. These results demonstrate that reliable MS analysis can be obtained in the presence of low levels of SDS. It is likely that protein/peptide recovery becomes a dominating factor in determining the number of identifications.

Table 2-4. MS identifications from low and high molecular weight GELFrEE fractions of *E. coli* proteins, following SDS depletion.

	Acetone		FASP		In common
	Total ID's #	Unique #	Total ID's #	Unique #	Total ID's # (%)
Protein					
Low MW	423	97	378	52	326 (69%)
High MW	256	47	226	17	209 (77%)
Peptide					
Low MW	2737	1225	2342	830	1512 (42%)
High MW	2769	1291	2317	822	1495 (41%)

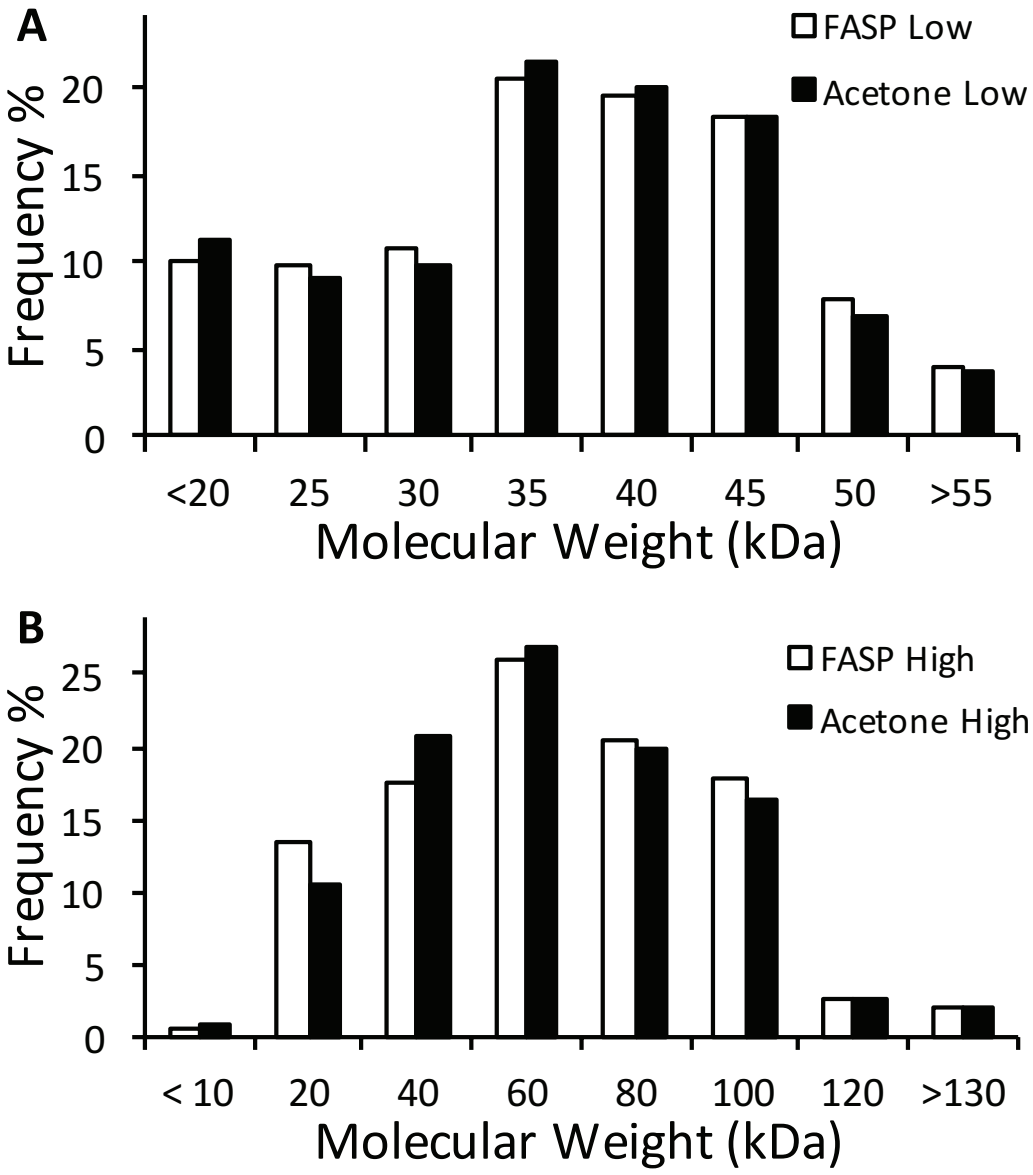

Figure 2-7. The distribution of proteins identified by mass spectrometry according to molecular weight as obtained from the low MW fraction **(A)**, and the high MW fraction **(B)** following SDS depletion. Note the differences in x-axis scales between low and high MW fractions.

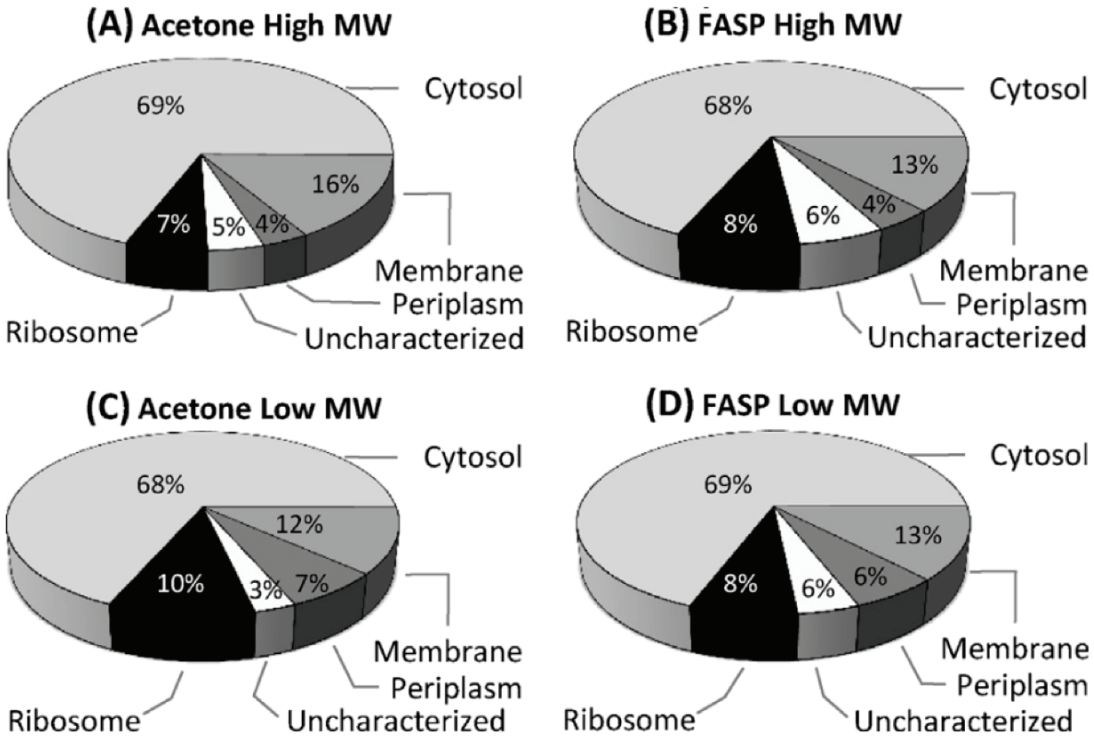

Figure 2-8. The cellular components of *E. coli* proteins identified by mass spectrometry, as obtained through Gene Ontology functional annotation by DAVID. The high MW fraction is shown in following SDS depletion in acetone precipitation **(A),** or using FASP **(B).** The low MW fraction is summarized in **(C)** and **(D)** following acetone or FASP purification, respectively.

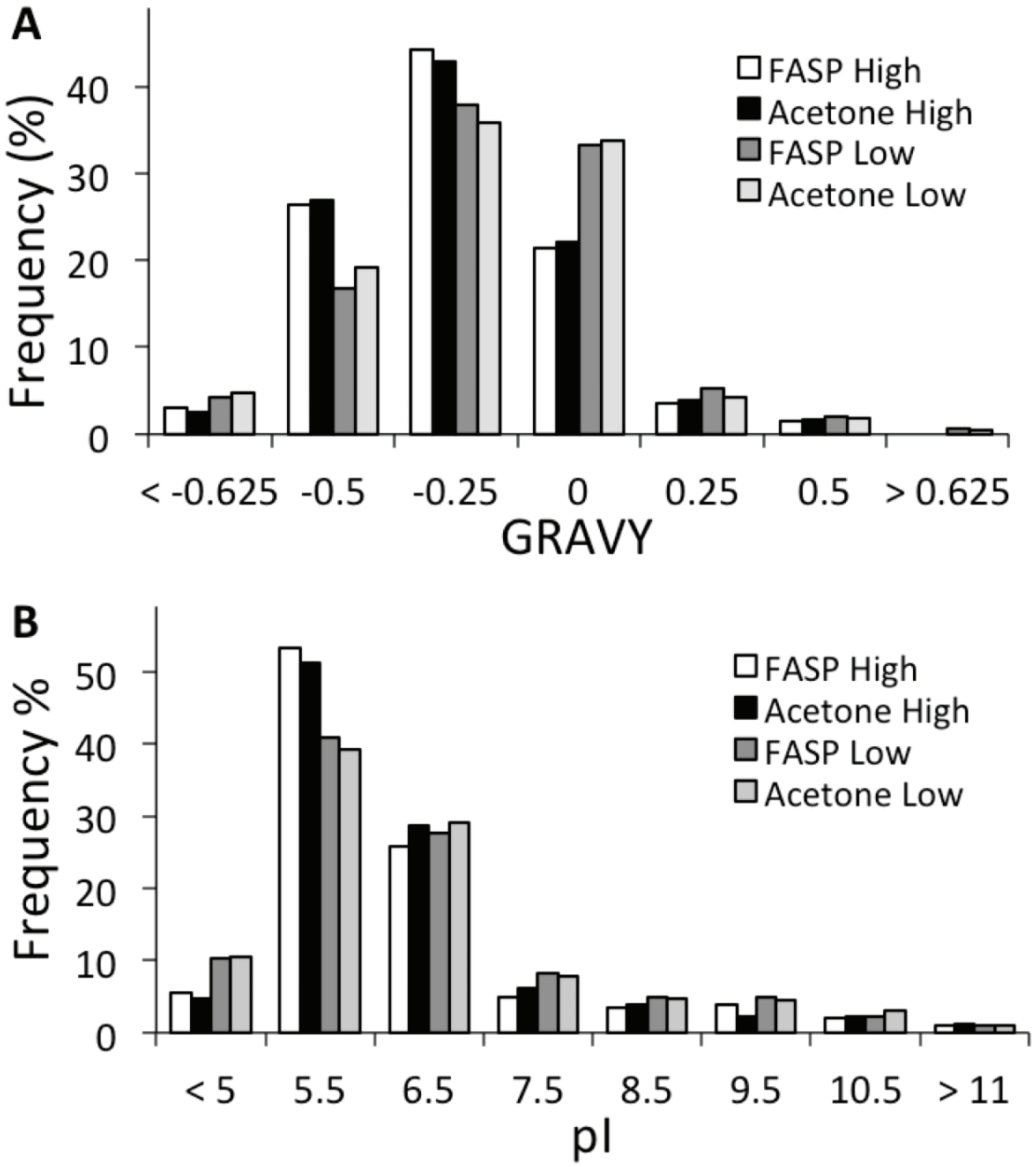

Figure 2-9. Distribution trends based on **(A)** hydrophobicity, represented by the Grand Average of Hydropathy (GRAVY) scores, and **(B)** isoelectric point (pI) for the proteins identified by mass spectrometry. Two GELFrEE fractions of *E. coli*, namely a low molecular weight (~ 30-45 kDa) and high molecular weight (~ 55-50 kDa) fraction, were subject to SDS depletion using either FASP or acetone precipitation. With GRAVY, hydrophobic proteins possess increasingly positive scores.

Despite no obvious trends in the types of proteins identified through FASP vs acetone precipitation, Table 2-4 demonstrates relatively low overlap in the identified proteins (69 and 77 %) and peptides (42 and 41 %) for the low and high MW GELFrEE fractions. The acetone precipitation protocol encompasses significant differences over FASP in terms of how proteins are processed. Unlike acetone, proteins should remain in solution throughout the FASP protocol, being concentrated as they are retained by the MWCO filter. Following acetone precipitation, our protocol relies on a combination of urea as well as the digestion step itself to resolubilize and recover peptides. Considering the molecular weight distribution of identified peptides, a trend was noted wherein larger molecular weight peptides were observed following the acetone precipitation protocol (Figure 2-10). Examining the number of missed cleavage sites for these peptides, the acetone protocol generates peptides with a higher degree of missed cleavages. From the list of uniquely identified peptides, 38 % of the peptides identified from acetone contained one or more missed cleavages. This doubled the number observed through FASP (19.4 %). An increase in the number of missed cleavages may also partially contribute to the larger number of peptide and protein identifications obtained through acetone precipitation.

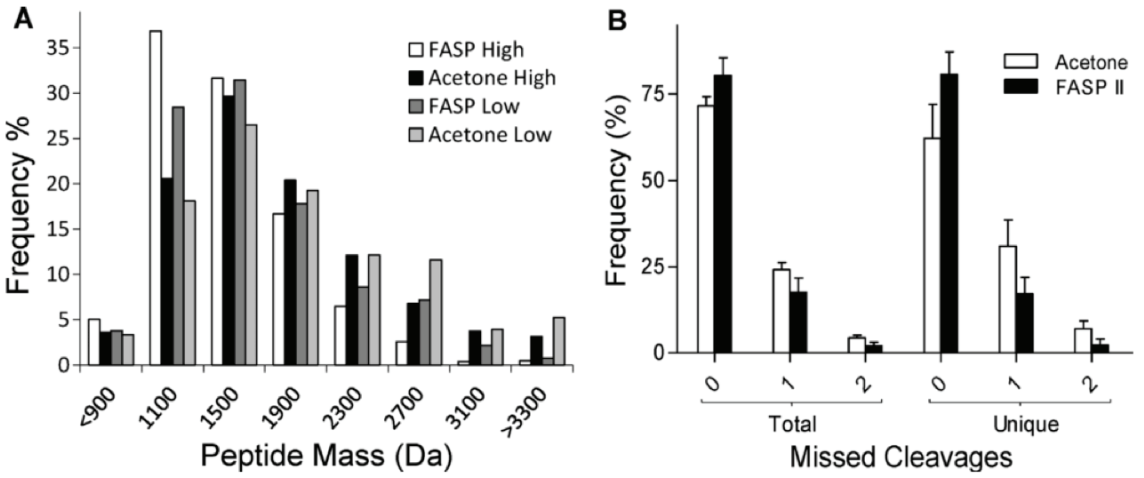

Figure 2-10. **(A)** Distribution of peptides identified by mass spectrometry according to their molecular weight, following detergent depletion of the low MW and high MW GELFrEE fractions. **(B)** Summarizes the relative abundance of trypsin missed cleavage sites found on the total list of identified peptides, as well as those peptides unique to the acetone precipitation or FASP depletion protocols.

With knowledge of the recovery and residual SDS expected from acetone and FASP, the MS results shown here are not unexpected. These results can be placed in context with previous assessments of various depletion strategies. Zhou *et al*[274] reported a similar number of protein identifications between samples subject to KCl precipitation relative to the FASP protocol. The study employed peptide-level SDS depletion, and so improved SDS depletion would readily permit MS analysis. The protein recovery values we observe for KCl at high concentration were similar to those of FASP, thus one might also expect a similar number of MS identifications. Given the sample complexity and the large amount of sample injected (2.5 μg), an MS method would not be sensitive to small changes in protein recovery. An independent study by Sharma *et al* compared FASP, TCA and chloroform precipitation, as well as Pierce detergent removal cartridges (protein level) for SDS depletion of GELFrEE fractions.[285] At high sample concentration, these

methods revealed similar numbers of MS identifications regardless of the protocol used. At low concentration however, FASP outperformed all other methods. While this is to be expected of the Pierce cartridges (poor recovery at low concentrations), the result contrasts with our own study comparing FASP to acetone precipitation. As shown previously,[12] high protein recovery is possible at low concentration, though a smaller pellet size increases the difficulty of the method. So long as precipitation is performed with high recovery, one would not expect a drop in MS performance.

2.4 Conclusion

With several choices available for SDS depletion, numerous factors (throughput, cost, versatility, etc.) will influence the choice of technique. Here the efficiency of a depletion strategy was quantified by considering only the purity of the analyte, together with the degree to which protein is recovered once the SDS is removed. While several methods can effectively deplete SDS to levels permitting MS analysis of proteins, all come with a cost in terms of analyte loss. Perhaps not surprisingly, in gel-digestion is extremely effective at removing SDS while recovering digested peptides in high yield. As a solution-based equivalent, FASP provides exceptional protein purity, but suffers considerable analyte loss. This translated into reduced protein and peptide identifications compared to an alternative approach of acetone precipitation. These results clearly demonstrate the capacity of incorporating SDS into a solution based proteomics workflow, and emphasize the importance of proper sample preparation to maximize detection of proteins by MS. However, knowing that challenges caused by improper pipetting techniques affect the recovery of acetone precipitation, particularly at low analyte concentration, the following chapter discusses the development of a novel method to deplete SDS.

Chapter 3: Development of Transmembrane Electrophoresis *

3.1 Introduction

In proteomics there exists a need for a SDS depletion method that is fast, reliable, and reproducible and that works with all types of protein samples. Chapter 2 compared the protein recovery and protein purity obtained from eight commonly used SDS depletion strategies. Several other popular methods of SDS depletion were omitted from this comparison (*e.g.* dialysis, SEC, HILIC, RPLC) because they are known to be ineffective at removing protein bound SDS. Though dialysis is ineffective at depleting bound SDS because it utilizes passive diffusion, filtration techniques such as FASP use centrifugal force to disrupt SDS-protein binding and wash away SDS while retaining protein atop a MWCO filter.[153] Other methods that have been used to remove SDS incorporate electrophoretic strategies such as electroelution or electroblotting, which utilize an electric potential to draw proteins out of SDS-PAGE gels.[174,305–308]

* A version of this chapter has been published in Kachuk, C., Faulkner, M., Liu, F., Doucette, A. Automated SDS depletion for mass spectrometry of intact membrane proteins through transmembrane electrophoresis. Journal of Proteome Research. (2016). DOI: 10.1021/acs.jproteo me.6b00199. Kachuk contributed to all experiments except the production of Figure 3-8 (Faulkner, M) and the initial voltage / current optimization (Liu, F). Doucette contributed the design of the device (Figure 3-3). Kachuk and Doucette contributed to the writing of the manuscript.

The application of an electric potential to move ions across a membrane is not a new concept in proteomics. In 1926, the Mattson electrodialysis cell was introduced as a way to pull ions through parchment paper towards an anode or a cathode.[309] This electrodialysis device was employed for a variety of uses including the precipitation of proteins,[310–312] but it was not until 1975 that Tuszynski and Warren use it at low current to lower the concentration of SDS from protein samples.[246] In this report, depletion of free and protein-bound SDS was achieved over an 11 hr period.[246] Following this paper, electrodialysis continued to be used to deplete SDS in combination with gel electrophoresis.[313,314] Also at this time, the term 'electrodialysis' became reserved for an entirely distinct process. Electrodialysis now refers to the transport of molecules through a series of semipermeable cation and anion exchange membranes (Figure 3-1).[315] This process has been used industrially since 1959 to desalinate water.[316] Besides desalination, other industrial electrodialysis applications include acid and base recovery and production,[317,318] table salt production,[319] and electrodialytic energy generation.[320] However, as with most filtering techniques, membrane fouling is an issue.[321] Regardless of terminology, the migration of SDS through a membrane filter with the aid of an electric potential as a front end tool for mass spectrometry has not been described in the literature.

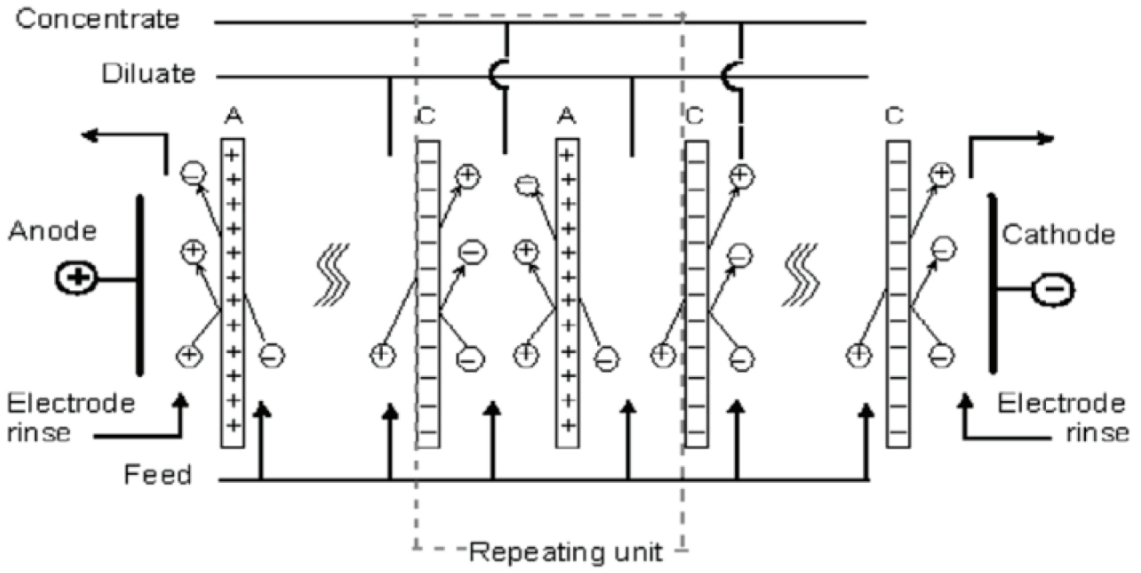

Figure 3-1. Schematic diagram illustrating the principle of 'modern day' electrodialysis. Figure reprinted with permission from reference [321]. Copyright Elsevier 2010.

The success of filtration (electrophoretically driven, or more conventionally by pressure) is often influenced by membrane fouling, giving rise to reduced analyte recovery.[147,322] For instance, the membrane filtration approach used by FASP is considered a form of dead-end filtration, where flow is perpendicular to the membrane.[323,324] Dead-end filtration of proteins may create particle – particle interactions and have an important influence on the success of filtration.[325–327] Proteins that are of similar size or only slightly smaller than the pores of the membrane will frequently plug the pores.[328] If proteins aggregate, this effect can be enhanced.[329] Larger proteins can also cause fouling by creating a gel layer on the surface, thus reducing the passage of smaller components through the membrane.[330] Even small proteins have the potential to deposit within the pores of the membrane and restrict passage.[331,332] Cross-flow filtration (solvent movement is par-

allel to the membrane) has been employed to decrease the amount of membrane fouling, particularly when applying an electric field.[333]

Cross-flow electro membrane filtration (aka electrofiltration) can circumvent membrane fouling by applying an electric field that moves charged particles away from the filter.[323,334] The applied electric field acts parallel to the flow direction of the filtrate but the high energy required results in heat production that compromises the protein residue.[325] By incorporating a continuous cross flow of the flush liquid (as shown in Figure 3-2), and with improvements made to residue recovery, the heat is able to dissipate sufficiently.[323,335] Electrofiltration has previously been applied to isolate and purify proteins, though SDS quantitation and subsequent MS analysis was not assessed.[336]

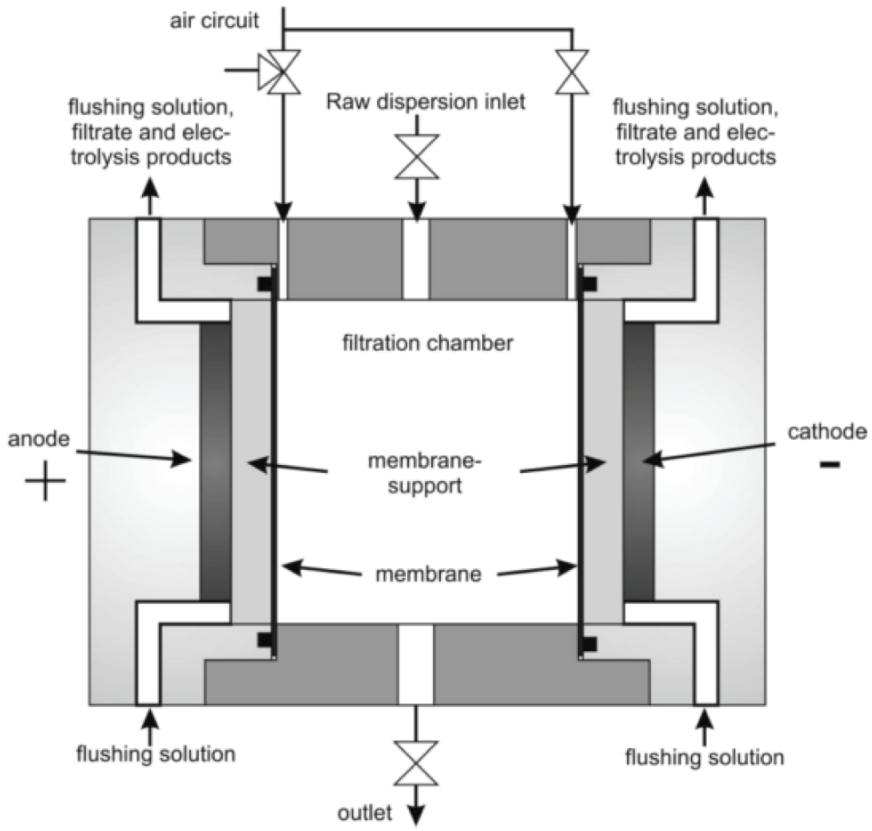

Figure 3-2. Scheme of the electrofiltration chamber. Figure reprinted with permission from reference [323]. Copyright John Wiley and Sons 2009.

In this work, a simple electrophoretic device for SDS depletion of membrane proteins is described. 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, neither dead-end filtration nor cross-flow are 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 is termed transmembrane electrophoresis (TME), offers a fully automated platform for SDS depletion ahead of LC-MS.

3.2 Experimental

3.2.1 Transmembrane Electrophoresis (TME) Design

A schematic of the TME device (machined by Mike Boutilier at Dalhousie University) is provided in Figure 3-3. 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.

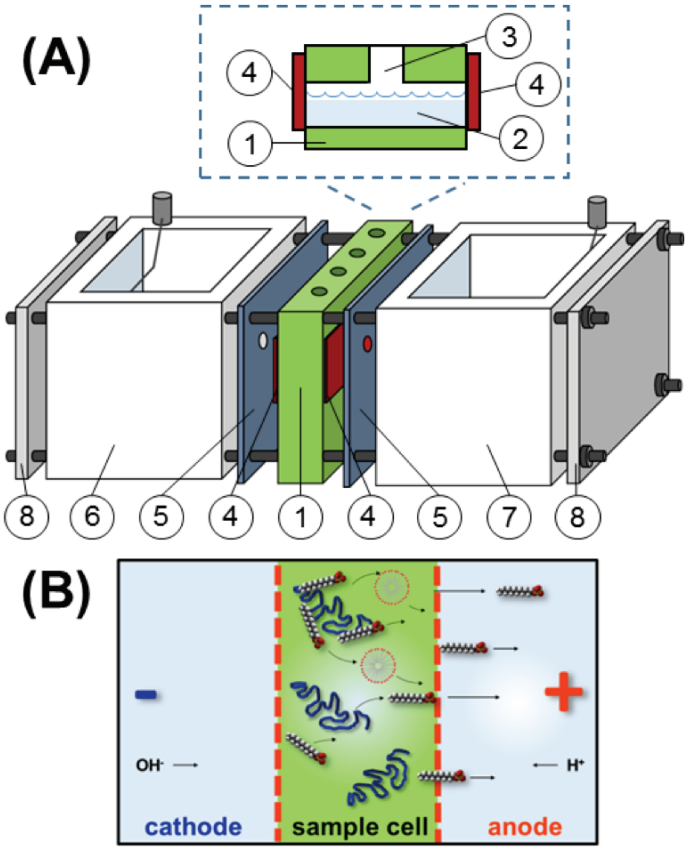

Figure 3-3. **(A)** An accurate model of the SDS depletion apparatus. The sample cell cartridge (1) comprises four discrete channels (2) with a sample inlet (3) bordered by MWCO filters (4). The wavy line depicts the height of the sample solution within the sample chamber. **(B)** Anionic SDS is driven 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 by the MWCO membrane. Device design was contributed by Alan Doucette.

3.2.2 Chemicals and Reagents

Bovine serum albumin (BSA), myoglobin, and TPCK-treated trypsin (T8802) were purchased from Sigma (Oakville, Canada). Milli-Q water was purified to 18.2 MΩ cm. Organic solvents (acetone, methanol, chloroform, acetonitrile, isopropanol) were of HPLC grade and obtained from Thermo Fisher Scientific (Ottawa, Canada). Methylene blue was also from Fisher. Reagents for casting and staining SDS PAGE gels, as well as urea, dithiothreitol (DTT), iodoacetamide (IAA), and SDS were from Bio-Rad (Mississauga, Canada). Formic acid (98%) was from Fluka (Mississauga, Canada), while trichloroacetic acid (TCA), trifluoroacetic acid (TFA), and all remaining chemicals used were from Sigma.

3.2.3 Sample Preparation

3.2.3.1 BSA and Myoglobin

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).

3.2.3.2 *E. coli* Growth and Membrane Protein Extraction

E. coli proteome extracts were obtained from a fresh cell culture, grown according to established protocols.[291] In brief, *E. coli* was grown in LB media at 37 ^{o}C with shaking until an OD_{600} of 0.7, and then harvested by centrifugation at 5,000 × *g* (15 min). To isolate the enriched membrane 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.[337] The enriched membrane 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.

3.2.4 SDS Removal

3.2.4.1 TME

To the assembled transmembrane electrophoresis (TME) device, 400 µL of SDS-containing protein solution was deposited into each of the four sample cells. The device was tested at multiple currents (0 to 50 mA) before 40 mA constant current was chosen as the optimal current, The device was run for one hour with periodic mixing of the sample by pipette throughout the run to prevent aggregation of the protein. SDS-depleted samples were then transferred to an Eppendorf vial. For SDS-depleted membrane 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,[293] 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.

3.2.4.2 Chloroform Methanol Water (CMW) Precipitation

Chloroform methanol water precipitation is as described previously by Wessel and Flugge,[338] with minor modifications.[164] Briefly, 400 µL of methanol, 100 µL chloroform, and 300 µL water were added to 100 µL of sample, with brief vortexing after each solvent addition. The sample was centrifuged (15 min, 21,000 × g) and the top layer was removed. A 400 µL aliquot of methanol was added, with gentle mixing to encourage the solvents to mix. The vial was centrifuged (15 min, 21,000 × g) and the solution was fully decanted. An additional wash was executed with 400 µL of methanol.

3.2.5 Proteome Analysis of *E. coli*

Following SDS depletion, 20 µL portions of the *E. coli* proteome fractions (whole cell or membrane enriched) were combined with 5 µL of Laemmli gel buffer,[144] 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,[235] 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.[237] 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 gradi-

ent 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.[339] 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 ± 5 ppm.

The enriched *E. coli* membrane 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 onto a self-packed 1 × 50 mm R2 column (Applied Biosystems), using a temperature programmed gradient described previously by Orton *et al*,[340] 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.

3.2.6 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.

3.2.7 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[341] partner repository with the dataset identifier PXD003941 and 10.6019/PXD003941. Cellular components were determined using Gene Ontology functional annotation from DAVID (Database for Annotation, Visualization, and Integrated Discovery).[120,121] Hydrophobicity was determined from GRAVY scores[127] obtained from www.bioinformatics.org/sm2/protein_gravy.html, while transmembrane topology was predicted using web-based software employing the TMHMM[108] and AmphipaSeeK[107] algorithms. ImageJ[342] 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.

3.2.8 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.

3.3 Results and Discussion

3.3.1 SDS Depletion

The principles of transmembrane electrophoresis are visualized in Figure 3-3B. SDS-containing protein solutions 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 voltage, 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 3-4, 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 3-4, 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.[164] 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.

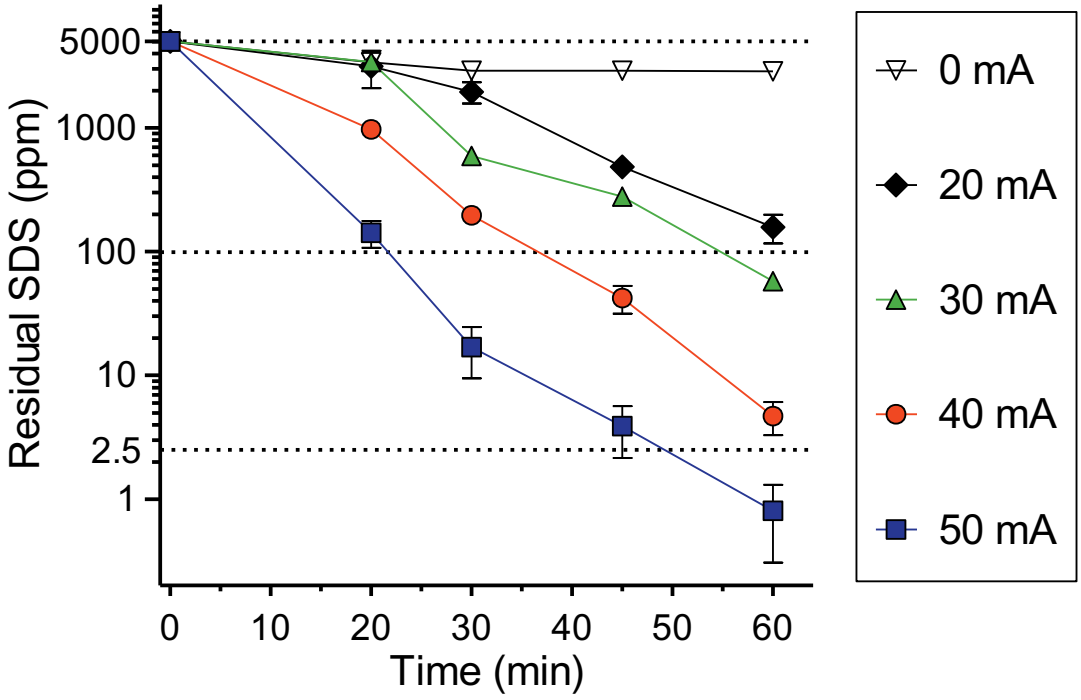

Figure 3-4. Time course of SDS depletion as a function of the applied current. Solutions initially comprised 150 µg BSA in 0.5 % SDS (5000 ppm). The critical value that permits LC-MS/MS analysis is indicated (100 ppm), as is the limit of quantitation of the methylene blue assay (2.5 ppm) used to monitor residual SDS. Error bars represent standard error of four replicates.

3.3.2 Protein Recovery

So long as the current did not exceed 40 mA, SDS depletion occured with minimal loss of analyte. BSA recovery was quantified above 90 % in all cases (Figure 3-5A). 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 34 ºC over the 1 hour experiment. At 50 mA the temperature rose to 60 ºC (Figure 3-5B). A high temperature increase is indicative of the high solution resistance as ions travers 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 electrofiltration.[333] 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.

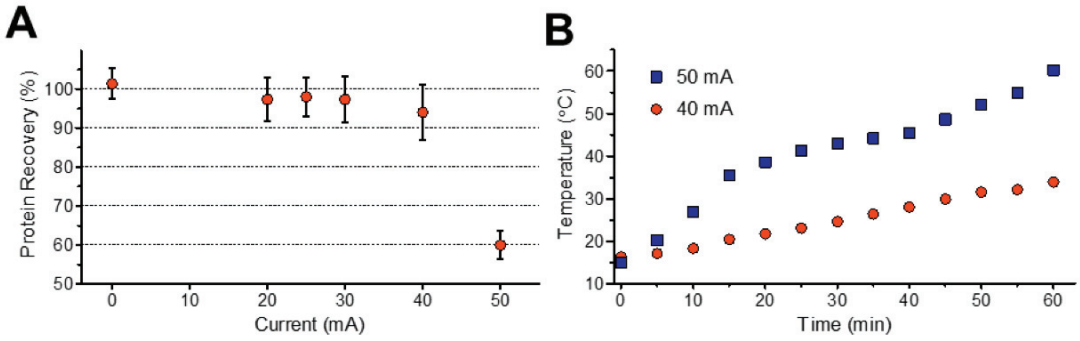

Figure 3-5. (A) The recovery of protein following one-hour SDS depletion using various currents as shown in Figure 3-4. Samples initially comprised 300 µL of 0.5 g/L BSA. Error bars represent standard error for depletion of four independent samples. **(B)** The temperature of the sample cell was monitored over the course of the SDS depletion experiments.

3.3.3 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 3-6 summarizes the results. Despite higher initial SDS, TME successfully removes the detergent to below 10 ppm (Figure 3-6A). This level of depletion compares favourably to other protocols for SDS depletion.[237] The protein recovery values are shown in Figure 3-6B. Withthe lower protein concentration samples, recovery was statistically indistinguishable from 100 % regardless of the initial concentration of SDS. Recovery was superior at these lower protein concentrations compared to the higher protein concentration though recovery remained above 90 % for all trials. This again compares favourably over alternative methods of SDS depletion.[237]

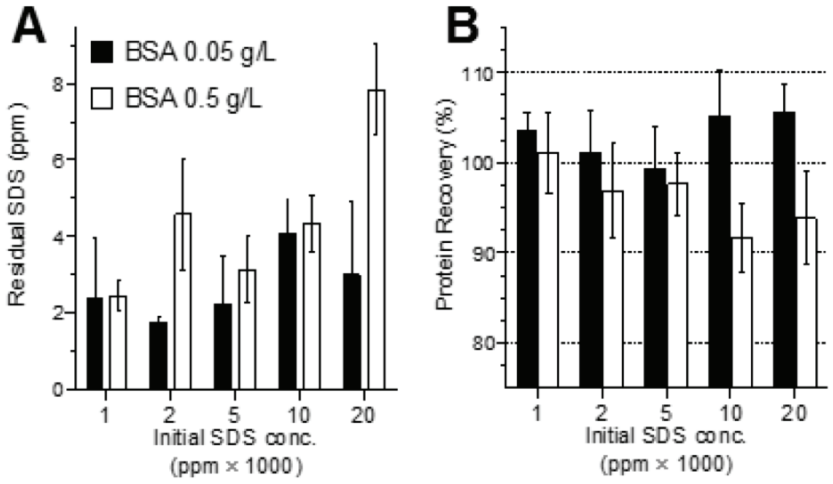

Figure 3-6. (A) Residual SDS and **(B)** protein recovery observed following SDS depletion (1 hour, 40 mA), as a function of the initial surfactant and protein concentration. Error bars represent standard error for depletion of four independent samples.

3.3.4 Mass Spectrometry of SDS-depleted proteins

Myoglobin was employed as a test sample and subjected to LC-MS analysis as the intact protein. As seen in Figure 3-7, though a charge envelope can still be obtained, a control solution containing 10 ppm SDS (Figure 3-7A) shows considerably less signal degradation compared to the 100 ppm spiked control (Figure 3-7B). The maximal tolerance of LC-MS towards SDS is not an absolute value and depends on the amount of protein being analyzed, together with the instrumental operating conditions. Acetone precipitation readily depletes SDS below 100 ppm.[164] As shown in Figure 3-7C, 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 3-7D). Based on these results, transmembrane electrophoresis is capable of removing SDS from protein samples to levels favourable for LC-MS analysis.

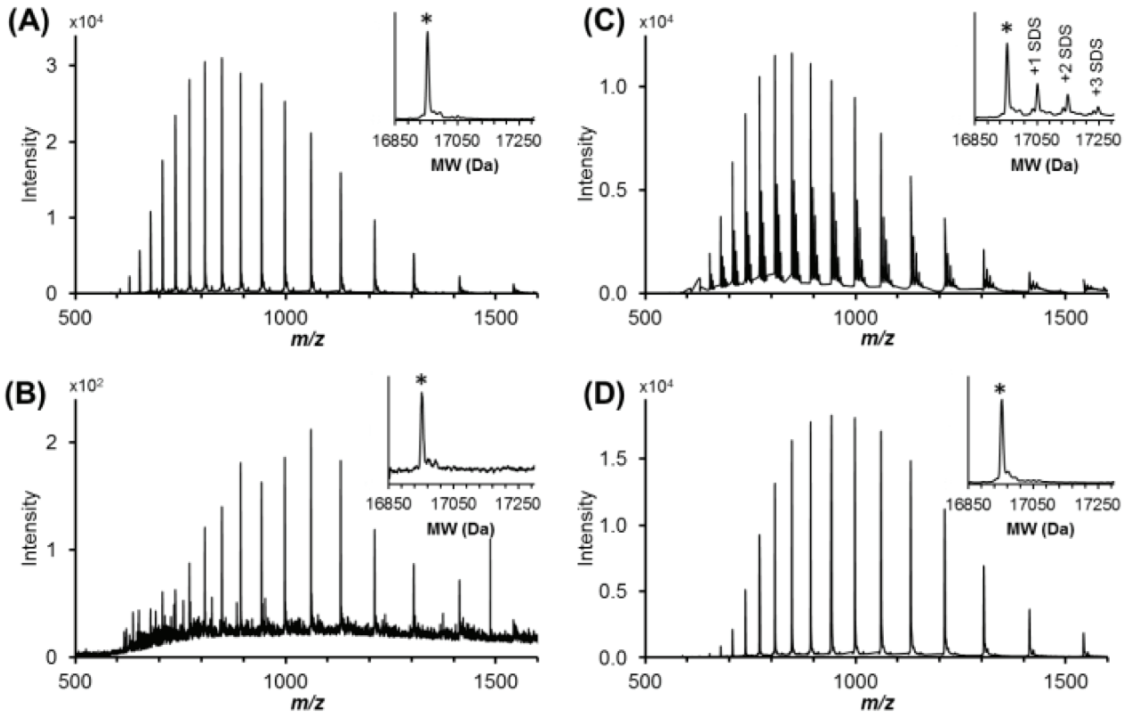

Figure 3-7. MS spectra of myoglobin, spiked with **(A)** 10 ppm SDS or **(B)** 100 ppm SDS. **(C)** SDS is depleted from the protein via acetone precipitation (initial 5000 ppm SDS). **(D)** SDS is depleted from an equivalent sample via transmembrane electrophoresis. Insets show the deconvoluted spectra, with the labelled peak (*) corresponding to the unmodified protein (16,951 Da).

3.3.5 Application to Proteome Analysis

Previously, M. Faulkner tested an *E. coli* 'whole cell' proteome extract spiked with 0.5 % SDS was employed 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 3-8 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 File S9. Examining the Venn diagram in Figure 3-8, 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 3-8 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, 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, Vieira *et al* have previously reported that trace levels of SDS (~10 ppm) can contribute a minor enhancement to MS signals for electrosprayed peptides.[165] It is also possible that denaturation 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.

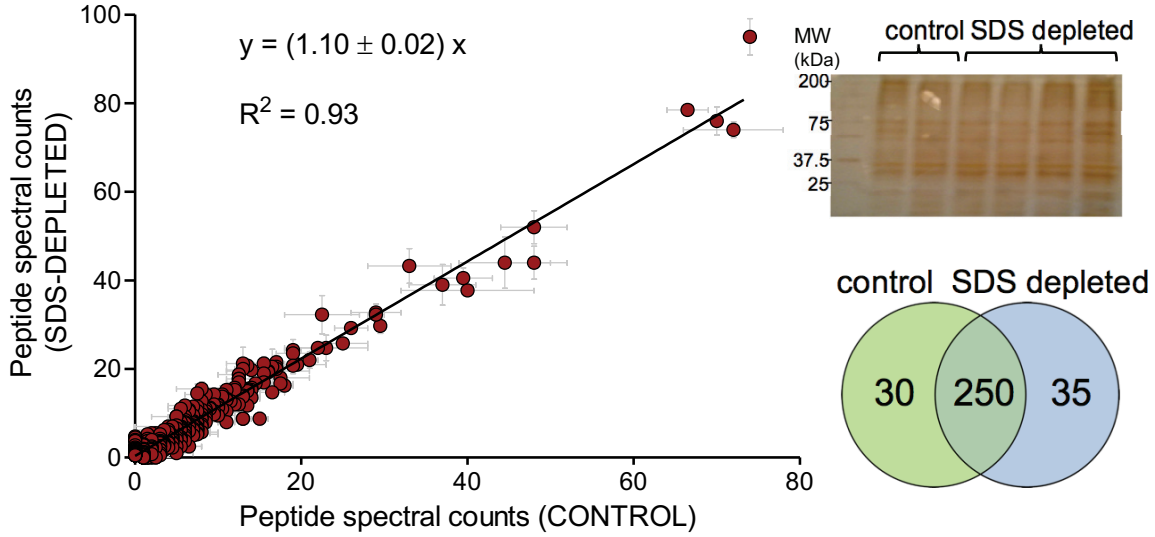

Figure 3-8. Comparison of proteomic data for SDS-depleted *E. coli* extract in 0.5 % SDS relative to a control, prepared in the absence of SDS. SDS PAGE (top right) shows the band intensity of the depleted fractions to be similar to the control. The Venn diagram (bottom right) summarizes the number of 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. * The data for this figure were previously obtained by Melissa Faulkner from the Doucette group.

3.3.6 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 membranes 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 recovery such proteins. Here, a rapid wash of the sample cell with cold (-20 °C) formic acid was utilized where Doucette *et al* has previously shown the effectiveness of this solvent to rapidly solubilize precipitated membrane proteins, being as effective as a solvent as employing 1 % SDS with extended sonication.[12] Maintaining a reduced temperature prevents protein formylation, which otherwise occurs when samples are exposed to formic acid.[12]

Figure 3-9 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 3-9), depending on the degree of protein aggregation that occurs in the absence of SDS. However as shown in Figure 3-9, 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. Some gel bands are of higher intensity in the water fractions (*e.g.* 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.

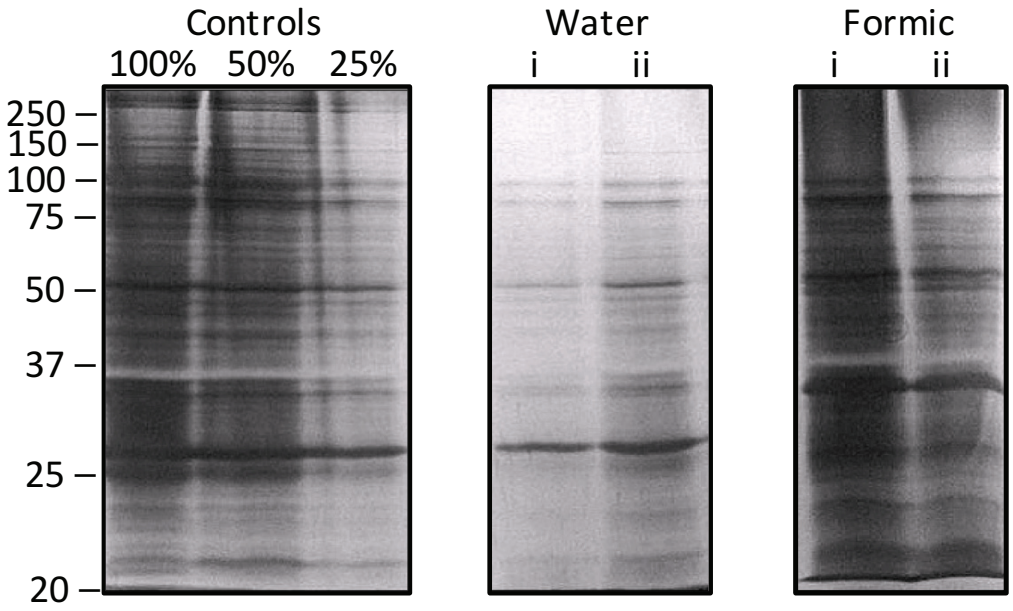

Figure 3-9. The *E. coli* membrane proteome extract as visualized by SDS-PAGE.[235] Controls are for the equivalent sample without TME purification and depict a hypothetical recovery of 100, 50, or 25 %. Proteins directly recovered in the 'water' fraction from independent sample replicates (i or ii) following TME are shown. The 'formic' shows the additional proteins that were recovered from the sample cells (i or ii), following a wash with 80 % cold formic acid.

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 File S10. 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.[12] 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 3-10). 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.[12] 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[108] and AmphipaSeek[107] algorithms. As shown in Figure 3-10B, 102 (57 %) contained in-plane membrane (IPM) anchoring points and 70 (39 %) contained transmembrane segments. Proteins with transmembrane segments are generally more hydrophobic than their counterparts.[147] 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, 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, 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.

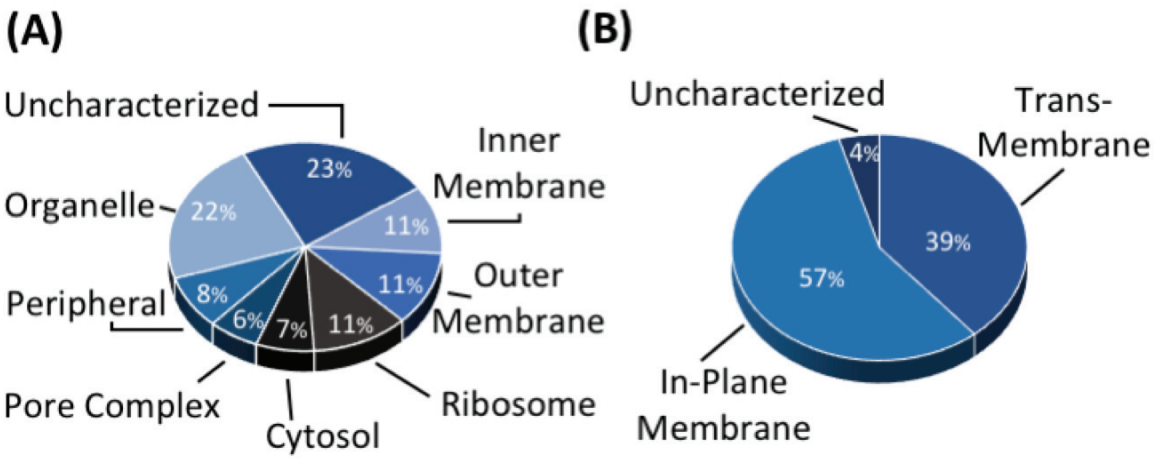

Figure 3-10. (A) The cellular compartments of the recovered proteins as profiled by the Gene Ontology function in DAVID. **(B)** All proteins, except those identified as ribosomal or cytosolic, were further assessed based on their interactions with the membrane and characterized as containing 1 to 16 transmembrane segments with TMHMM,[108] or 3 to 42 in-plane membrane (IPM) anchors identified with AmphipaSeek.[107]

3.3.7 Mass Spectrometry of Intact Proteins

One of the significant advantages of TME is its amenability to top down work-flows. LC-MS analysis of intact proteins recovered from these same *E. coli* membrane protein fractions following TME purification was also demonstrated. Figure 3-11A 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 Figure 3-12). 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.[164] This TIC trace is shown in Figure 3-11B. 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), the most abundant membrane protein in *E. coli*. Deconvolution of the charge envelopes reveals several common proteins detected across the three fractions, though some masses were uniquely detected (Table 3-1). Regardless of the sample purification approach, no SDS adducts were observed. Furthermore, the use of cold formic acid preserved the unmodified mass of the protein, as formylation events (+28 Da) were also not detected (Figure 3-13).[12] The distinct charge state envelopes observed with high signal-to-noise ratio for multiple proteins demonstrates the ability to incorporate TME into an intact protein workflow.

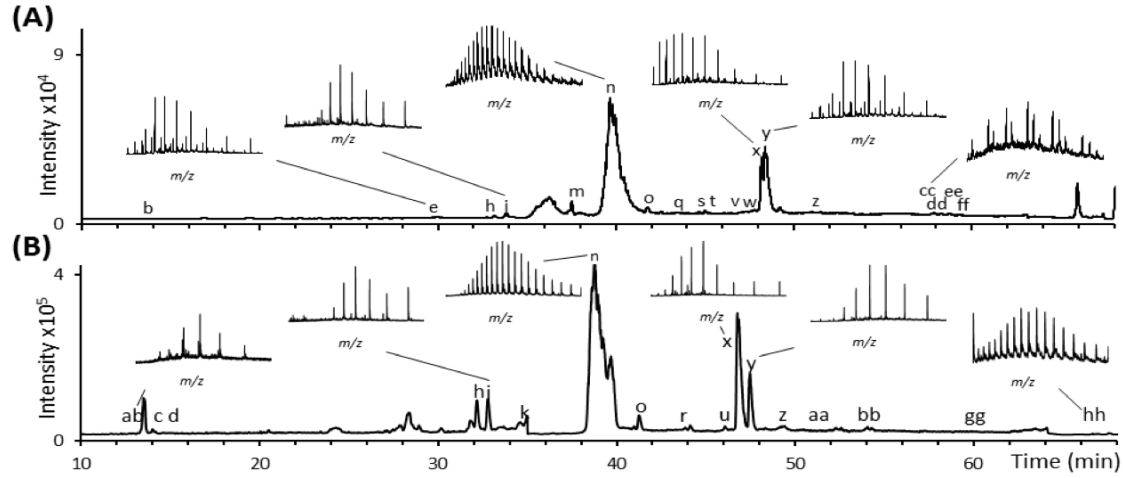

Figure 3-11. TIC traces for *E. coli* membrane extracts from analysis of **(A)** the formic acid fraction recovered from TME, and **(B)** CMW precipitation. Letters correspond to distinct charge state envelopes. A selection of spectra is shown (deconvoluted as insets). A complete list of observed proteins is provided in Table 3-1.

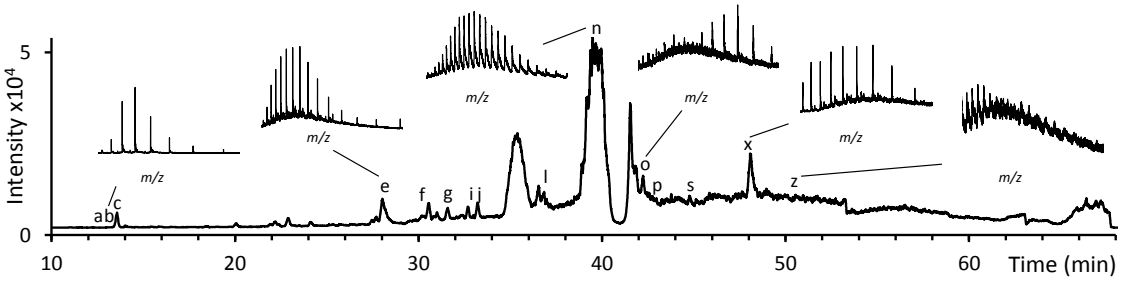

Figure 3-12. TIC trace and associated mass spectra (insets) of selected peaks detected in the water fraction after SDS depletion with transmembrane electrophoresis. The letters correspond to all observed proteins, with the complete list of masses found in Table 3-1.

Table 3-1. Intact *E. coli* membrane proteins identified in TIC traces.

Peak Number	CMW		Formic		Water	
	Mass	±	Mass	±	Mass	±
a	8737	2	-	-	8738	0.3
b	8605	1	8606	1	8606	2
c	9318	1	-	-	9318	3
d	9553	3	-	-	-	-
e	-	-	11190	2	11188	2
f	-	-	-	-	5384	1
g	-	-	-	-	8801	2
h	10403	2	10403	2	-	-
i	10385	1	10388	2	10388	4
j	-	-	-	-	7623	2
k	13022	2	-	-	-	-
l	-	-	-	-	15503	1
m	-	-	7334	1	-	-
n	35168	5	35165	11	35197	10
o	16397	1	16401	5	16399	1
p	-	-	-	-	15003	2
q	-	-	15774	3	-	-
r	23351	1				
s	-	-	9244	1	9228	1
t	-	-	23339	3	-	-
u	11105	2	-	-	-	-
v	-	-	7713	1	-	
w	-	-	11362	3	-	
x	10922	2	10924	2	10925	2
y	11175	2	11176	2	-	
z	35001	8	35015	10	35009	8
aa	18600	3	-	-	-	-
bb	18584	2	-	-	-	-
cc	-	-	12254	1	-	-
dd	-	-	12518	1	-	-
ee	-	-	12223	2	-	-
ff	-	-	12487	1	-	-
gg	38436	2	-	-	-	-
hh	35216	10	-	-	-	-

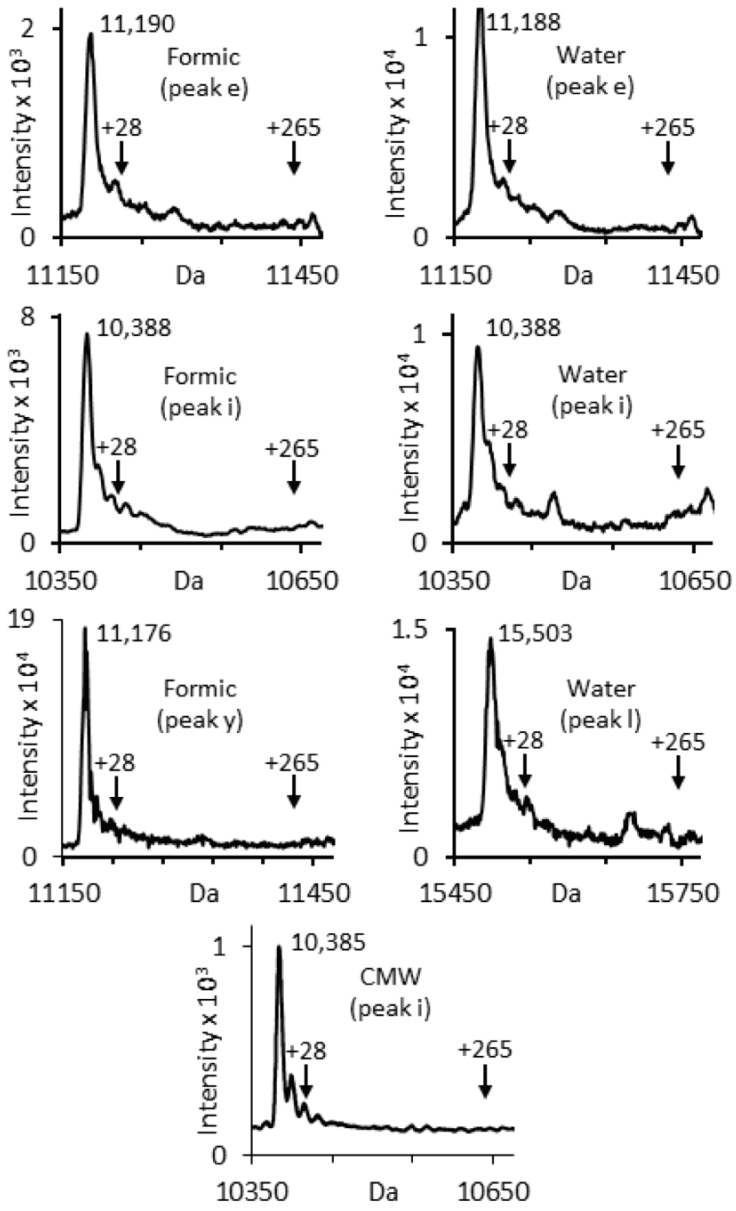

Figure 3-13. Deconvoluted spectra of *E. coli* membrane samples recovered from the formic fraction (left), the water fraction (right), and CMW (bottom), showing the absence of SDS and carbonyl adducts. Letters are associated with chromatographic features found in Figures 3-11 and 3-12, with a complete list provided in Table 3-1.

3.4 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).

Chapter 4: Improvement of Transmembrane Electrophoresis

4.1 Introduction

As seen in Chapter 3, transmembrane electrophoresis (TME) is a simple and effective technology that depletes SDS from protein samples to levels favourable for mass spectrometry analysis, without compromising protein recovery. The system utilized 40 mA constant current over a one-hour period to deplete SDS by overcoming the SDS-protein binding energy. SDS depletion by TME is particularly suited for top-down MS based sample preparations, and towards analysis of membrane proteins. Our ultimate goal for TME is to incorporate the device into an online system, where the sample would flow directly from a GELFrEE separation, through a TME purification step, to the LC-MS. To advance TME to the next phase of development, the rate of SDS depletion needs to improve. There are many parameters than can influence the rate of SDS depletion including, but not limited to: the pore size of MWCO dialysis membrane (Section 4.3.1); the temperature of the sample (Section 4.3.2); and the wattage applied to the system (Section 4.3.3). Early TME experiments initially employed constant voltage for SDS depletion. While higher voltage improved the rate of SDS depletion, protein recovery was compromised (data not shown). Constant current was therefore chosen (Chapter 3) to provide better control over the heat generated from Joule heating. It is noted that the resistance in the cell is not constant, and decreases over the course of a run (5.0 kΩ initial drops to 3.6 kΩ at 60 min). Consequently, the voltage also changed over the course of the run (200 V initial drops to 145 V at 60 min). Another mode of operation would be to maintain constant wattage. In doing so, we gain precise control over the heat generated in

the system ($Q = I^2 R$). Operating at constant wattage, current will increase over the course of the run, while voltage will decrease (resistance again drops). In essence, the applied voltage is tuned in response to the resistance of the system, such that the rate of SDS depletion is maximal.

4.2 Experimental

4.2.1 Transmembrane Electrophoresis (TME) Design

The TME apparatus described in Chapter 3 was used for these experiments. The power supply was changed to a Bio-Rad Power Pac 3000, which is capable of delivering constant power.

4.2.2 Chemicals and Reagents

Bovine serum albumin (BSA), myoglobin, carbonic anhydrase, cytrochrome C, lysozyme, and TPCK-treated trypsin (T8802) were purchased from Sigma (Oakville, Canada). Milli-Q water was purified to 18.2 MΩ cm. Organic solvents (acetone, methanol, chloroform, acetonitrile, isopropanol) were of HPLC grade and obtained from Thermo Fisher Scientific (Ottawa, Canada). Methylene blue was also from Fisher. Reagents for casting and staining SDS PAGE gels, as well as urea, dithiothreitol (DTT), iodoacetamide (IAA), and SDS were from Bio-Rad (Mississauga, Canada). Formic acid (98%) was from Fluka (Mississauga, Canada), while trichloroacetic acid (TCA), trifluoroacetic acid (TFA), and all remaining chemicals used were from Sigma.

4.2.3 Protein Sample Preparation

Protein solutions consisting of bovine serum albumin (BSA), myoglobin, cytochrome C, carbonic anhydrase, or lysozyme (Sigma, Oakville, Canada) were prepared in Milli-Q grade water, and containing the appropriate concentration of SDS (Bio-Rad).

4.2.4 TME Operation (Optimized)

Before assembling the device, regenerated cellulose dialysis filters with nominal MWCO between 1 and 14 kDa (Fisher Scientific, Ottawa, Canada) were hydrated in water for a minimum of 30 minutes before being positioned on either side of the sample cartridge. The device was then sealed by custom gaskets cast from Sylgard® 184 silicone elastomer (Dow Corning Corp, Midland, MI), and filled with deionized water (DI) to ensure the device is water tight. To the assembled device, 300 mL of chilled (4 ºC) electrolyte (25 mM Tris / 192 mM glycine, pH 8.3) was added to the buffer chamber, while ensuring that no air bubbles became trapped near the dialysis membrane. Protein samples (0.1 g/L in 0.5 % SDS) were also chilled to 4 ºC before loading 400 µL (40 µg) into each of the four sample cells of the device. The device was run at constant power.

When testing the MWCO membranes, the TME device was operated at a constant current of 50 mA. The application of power was paused briefly at 0, 5, 10, 20, 40, and 60 minutes to collect aliquots for residual SDS, and to collect temperature measurements. SDS was quantified using MBAS assay as described in Section 4.2.5. The aliquots were acquired following mixing of the sample by pipette (3 × 150 µL). When doing the time course experiment with small proteins and a 3.5 kDa MWCO membrane, aliquots for protein recovery were also taken during these pauses. Protein recovery was measured by BCA assay as described in Section 4.2.6

To find the optimal operation of TME, the device was operated at constant powers ranging from 8 W to 36 W. The application of power was paused briefly at 0, 5, 10, 20, 40 and 60 minutes to collect aliquots for a residual SDS and to collect sample chamber temperature measurements. At 24 W and higher, the TME device was additionally paused every minute for the first ten minutes to mix the samples by pipette (3 × 100 μL). The mixing was done to prevent aggregation of the protein against the membrane and to wash the walls of the inlet of the sample chamber to remove any high concentrations of SDS that were present as a droplet in the inlet. These pauses were brief (~ 15 seconds for the mixing pauses and 30 seconds for mixing and sample collection pauses). SDS was quantified using MBAS assay as described below in Section 4.2.5. The aliquots were acquired following mixing of the sample by pipette (3 × 100 μL). When the residual SDS was approaching less than 10 ppm, a further 50 μL, or 5 μg assuming 90 % recovery (original TME recovery) was removed for LC-UV clean-up and digestion, as described in Section 4.2.7.

Optimal operation based on the results presented here employs 36 watts over the following period: Apply power for 1 min, then pause until the sample cell temperature drops below 30 °C (~ 1 min). Repeat the power application then pause cycle until a total of 10 minutes of applied power has been acquired (~ 20 minutes total run time with pauses). During the 'pause' phase, the sample solution was mixed within the chamber by repeated pipetting (3 × 150 μL for each chamber). SDS depleted samples were then transferred to an Eppendorf vial where 100 μL was tested for residual SDS using MBAS. A further 50 μL, or 5 μg assuming 90 % recovery (original TME recovery), was removed for LC-UV clean-up and digestion as described in Section 4.2.7.

4.2.5 SDS MBAS Assay

The methylene blue active substances assay (MBAS) was performed as described by Arand *et al*.[293] In brief, a 100 µL aliquot of appropriately diluted sample was combined with 100 µL of methylene blue reagent (250 mg methylene blue, 50 g sodium sulfate, 10 mL sulfuric acid diluted to 1.0 L), vortexed briefly, then mixed with 400 µL chloroform. The samples were vortexed and centrifuged (1500 × *g*), retaining the lower chloroform layer for absorbance measurement at 652 nm on an Agilent 8453 spectrophotometer (Mississauga, Canada). Samples were run against a calibration curve ranging from 2 to 15 ppm.

4.2.6 Bicinchoninic Acid (BCA) Assay

Intact protein recovery was determined through a Pierce™ BCA protein assay. Aliquots of 15 µL was combined with 300 µL of BCA working reagent. Samples were heated in a 57 ºC water bath for 30 min, then cooled to room temperature prior to recording the absorbance at 562 nm. BSA was used to construct a calibration curve for all test samples.

4.2.7 LC/UV Assay and Clean-up

Following SDS depletion, 50 µL (approximately 5 - 10 µg) of sample were injected onto a self-packed 1 × 100 mm C18 column (Waters ODS 5 µm beads) using an Agilent 1200 high performance liquid chromatography (HPLC) series instrument. After a five minute hold, peptides were eluted as a single fraction through an instantaneous ramp from 5 % to 80 % acetonitrile, with the resulting UV absorbance signal monitored at

214 nm against a calibration curve consisting of BSA.[295] The collected fractions dried in a SpeedVac and stored at -20 ºC.

4.2.8 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.

4.3 Results and Discussion

4.3.1 TME and MWCO Membranes

4.3.1.1 SDS Depletion

In Chapter 3, the TME device was operated with a 3.5 kDa MWCO dialysis membrane to successfully deplete SDS from BSA and *E. coli* samples. Though the *E. coli* proteome contains proteins with small molecular weights (< 30 kDa), the effectiveness of removing SDS from smaller proteins was not directly evaluated. Thus, a series of time course experiments were conducted to deplete 0.5 % SDS from cytochrome C (12 kDa)[343], lysozyme (14 kDa)[344], myoglobin (17 kDa),[345] and carbonic anhydrase (29 kDa).[346] The TME device was operated with a 3.5 kDa MWCO membrane over a one-hour period at 40 mA. As shown in Figure 4-1, all samples were successfully depleted of SDS to levels below 10 ppm. Interestingly, SDS was depleted faster for carbonic anhydrase and lysozyme (within 40 minutes) whereas cytochrome C and myoglobin required the full 60 minutes. There does not appear to be a trend correlating SDS depletion time to the MW of the protein, as carbonic anhydrase and lysozyme are the largest and the second smallest proteins of the group, respectively. This result may be explained

by considering a different aspect of the way SDS binds to protein. In Section 1.3.3, the different rates of SDS-protein binding were discussed, highlighting the importance of the type of protein. Myoglobin in particular requires a multi-step process to reach binding equilibrium with SDS. The facilitated depletion of SDS from lysozyme compared to that of myoglobin may be attributed to their relativebinding mechanisms with SDS-.[347,348] While certain proteins could have been depleted of SDS in a shorter time, it is advised that TME purification continue to operate for one-hour, so as to ensure that all samples are properly purified.

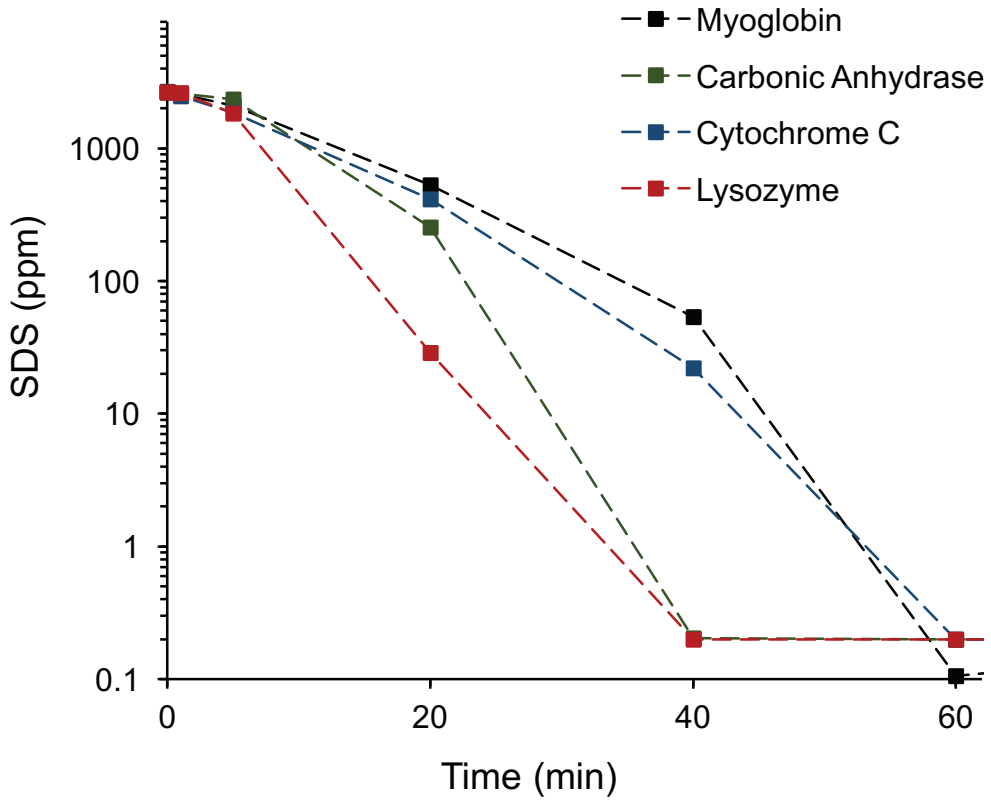

Figure 4-1. Time course of SDS depletion of from 400 µL of 0.1 g/L cytochrome C, lysozyme, myoglobin, and carbonic acid in 0.5 % SDS (5000 ppm), following TME purification. SDS content was as measured by MBAS assay. The LOQ of the MBAS assay is 2 ppm, thus the values presented below this are below the limit of quantitation and are solely presented for visualization.

The pore size of the dialysis membrane used may play a critical role in the time required to deplete SDS. Utilizing a larger pore size could theoretically allow for quicker SDS depletion, because the effective diameter restricting passage is larger.[349,350] To assess TME's amenability to deplete SDS with different pore sizes of MWCO dialysis membranes (1, 3.5, and 6-8 kDa), a series of time course experiments were conducted (Figure 4-2). Despite the predictions of increased SDS depletion with larger MWCO membranes, there was no such trend observed. The least effective MWCO membrane for SDS depletion was 3.5 kDa, though it still reduced SDS to 7 ± 9 ppm. The 1 and 6-8 kDa membranes reduced SDS to 1.0 ± 0.2 and 1.3 ± 0.4 ppm, respectively. When considering these results, it is important to keep in mind that the MBAS assay used has a LOQ of 2 ppm (Table 2-2) and that the data presented in the figure is displayed on a log scale. In absolute terms, the depletion of SDS from all three membranes achieves > 99.9 % depletion. Interestingly, the 1 kDa and the 6-8 kDa MWCO membranes attained sufficient SDS depletion within 40 minutes. The 3.5 kDa membrane however, took 60 minutes to deplete SDS. With no systematic patterns to be extracted from the data in Figure 4-2, it is difficult to surmise why the different pore size dialysis tubing behaved as they did. The age of the dialysis tubing, a contaminant in the sample or the buffer, an air bubble present against the dialysis membrane, or even standard error could have played a role in the results. Regardless, when considering both Figure 4-1 and Figure 4-2, SDS depletion consistently reaches levels compatible with MS analysis in one-hour.

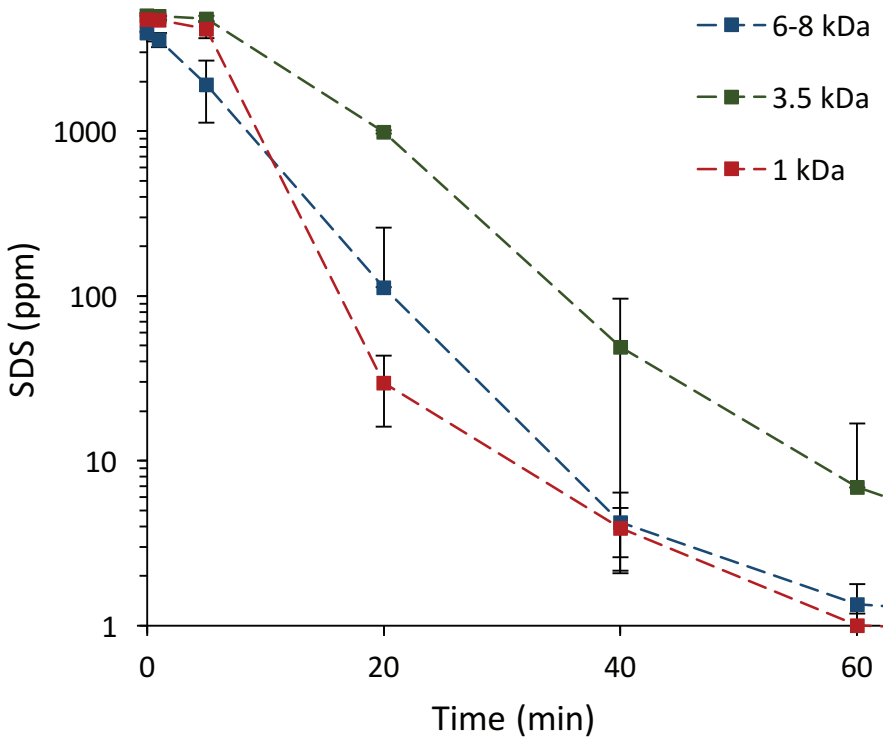

Figure 4-2. Time course of SDS depletion using transmembrane electrophoresis with 1, 3.5, or 6-8 kDa MWCO dialysis tubing. Solution initially comprised 40 µg of BSA in 0.5 % SDS (5000 ppm) in 400 µL. Error bars represent standard error of four replicate aliquots.

4.3.1.2 Protein Recovery

The use of MWCO membranes to retain the protein samples may present a risk of sample loss, particularly for smaller proteins or peptides.[269] Previously, a 3.5 kDa MWCO membrane filter was employed. However, the reported size cut-off for a dialysis membrane is not an absolute value, but represents a hypothetical macromolecular retention of 90 % for those molecules above the stated molecular weight. The apparent pore size of the dynamic membrane are affected by: (1) the type and state of the proteins present;[351] (2) changes in the physical size of the protein due to changes in pH or ionic strength;[352,353] (3) increased compaction or thickness due to increasing transmembrane

pressure;[354] (4) aggregation of protein at the membrane surface;[355] (5) membrane material,[356] membrane hydrophobicity,[357–360] or membrane porosity and heterogeneity.[361]

The dialysis process is also purely driven by concentration, whereas TME is driven by the electric potential across the membrane. As a consequence, smaller proteins approaching the size of the membrane are suspected to be more likely to pass through the membrane and be lost. This necessitates a direct evaluation of the effectiveness of dialysis membrane of varying pore sizes to retain proteins of lower molecular weight.[362] Beyond the risk of proteins passing straight through the membrane, sample loss may also occur through aggregation of proteins as they bind to the membrane (*i.e.* membrane fouling).[147,322,328,333] Particularly, following SDS depletion, the hydrophobic amino acids of fully-denatured proteins would tend to aggregate towards one another leading to precipitation. Adsorption onto the membrane itself can also provide a surface for initial aggregation of proteins. With the potential for protein loss when using TME, protein recovery of BSA was assessed using MWCO filters of various pore sizes (Figure 4-3). The smallest MWCO membrane (1 kDa) retained 86 ± 17 % BSA, while the largest pore size (14 kDa) retained 74 ± 2 % BSA. Although visually it appears that an increase in the pore size results in decreased recovery, a one-way ANOVA ($\alpha = 0.05$) indicates there is no significant difference between the means ($F_{3,23} = 0.13$, $p = 0.95$). Thus it can be concluded that changing the dialysis membrane to a larger or smaller pore size does not have a detectable effect on the recovery of BSA. BSA is a fairly large protein (66 kDa), so this is not surprising, but the pore size may impact the recovery of smaller proteins.

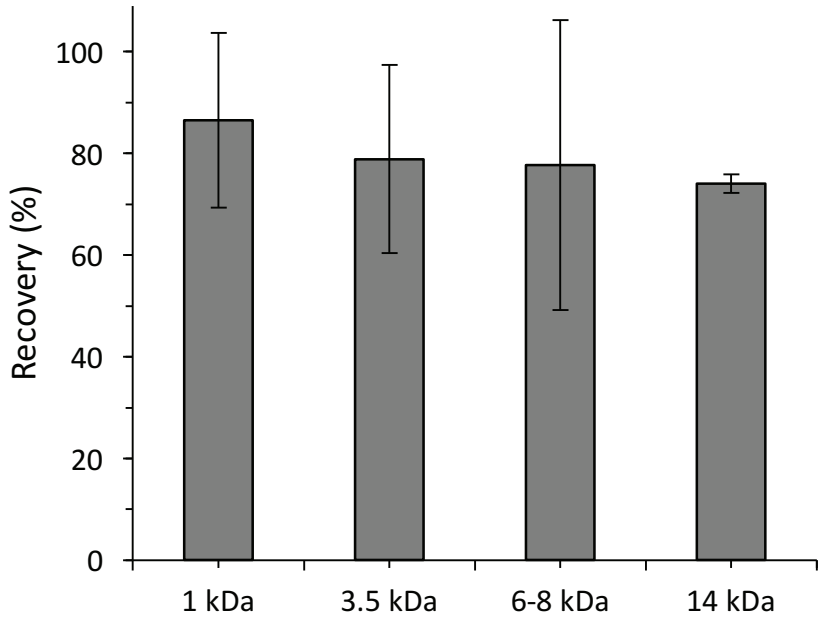

Figure 4-3. Protein recovery of 40 µg of BSA in 400 µL of 0.5 % SDS following TME purification using varying pore sizes of dialysis membranes. Data were collected over various runs. Error bars represent standard error of mean. n = 4 for 1 kDa; n = 24 for 3.5 kDa; n = 64 for 6-8 kDa; n = 8 for 14 kDa.

Given that current top-down instrumentation technologies are most compatible towards proteins that are less than 30 kDa,[84] the retention of smaller proteins is necessary to ensure TME's amenability to top-down MS-based protein identification workflows. To assess the effectiveness of TME at retaining small proteins (< 30 kDa), protein recovery following one-hour TME purification for cytochrome C, lysozyme, myoglobin, and carbonic anhydrase is found in Figure 4-4. In contrast to the high recovery observed with BSA, cytochrome C and lysozyme, the two smallest proteins, only retain 40 and 28 % of their initial concentration, respectively. The two larger proteins, myoglobin and carbonic anhydrase retained greater than 70 % of their initial concentration, but this was still less than that observed with BSA. It was expected that by using a 3.5 kDa MWCO mem-

brane, which is significantly smaller than that of the cytochrome C (12 kDa) and of lysozyme (14 kDa), the proteins would be recovered from the sample chamber. This was not observed here, where it is possible that: (1) the proteins in question are aggregating and binding to the dialysis membrane;[328] (2) the temperature of the sample cell is increasing the porosity of the membrane, allowing larger macromolecules to pass through;[363] or (3) the denatured proteins, which are more linear than globular, are passing through the dialysis pores end first.

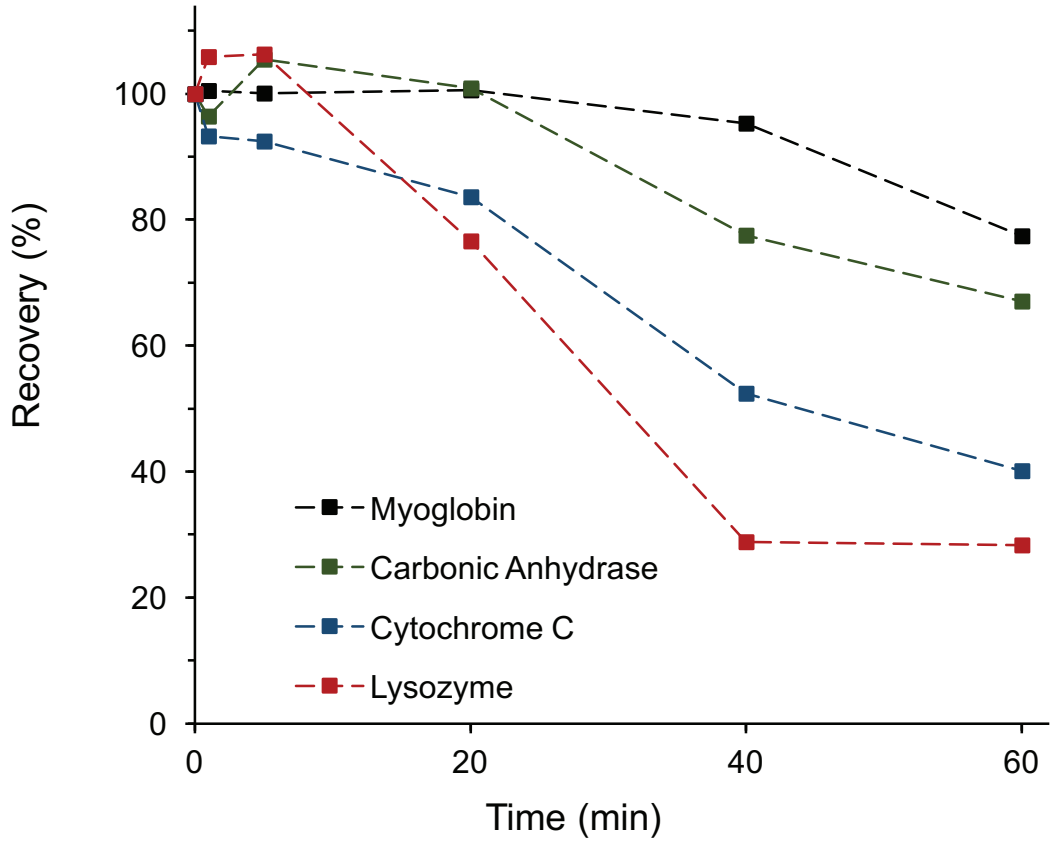

Figure 4-4. Time course of protein recovery of 40 µg of cytochrome C, lysozyme, myoglobin, or carbonic anhydrase in 400 µL of 0.5 % SDS (5000 ppm) during TME purification.

The hypothesis of protein aggregation being responsible for protein loss with cytochrome C and lysozyme is not explored here, but would be reconciled with inclusion of a cold formic acid wash, as described in Chapter 3. If, on the other hand, reduced protein recovery is caused by an increase in temperature expanding the pore size of the dialysis membrane, or if the denatured proteins are passing through on end, reducing the size of the MWCO membrane used should increase recovery. Therefore, by employing a 1 kDa membrane instead of a 3.5 kDa membrane, protein recovery should increase. Figure 4-5 shows the protein recoveries for BSA, myoglobin, carbonic anhydrase, cytochrome C, and lysozyme following TME purification using both 1 and 3.5 kDa membranes. As was seen in Figure 4-3, the recovery of BSA is not significantly changed with different membranes. This is likely because it is large enough to avoid the effects of what it would consider small changes in the size of the pores in the membrane. All four proteins under 30 kDa showed a 20 – 30 % increase in recovery. It remains unclear why the size of MWCO dialysis membrane used would make such a difference, given the smallest protein tested was 12 kDa and should not have been lost through the pores of the 3.5 kDa membrane. However, it is quite obvious that it does play a role, particularly with smaller proteins. Thus, despite the reported size cut-off stated by the manufacturer it is clearly important to choose an appropriate MWCO membrane when operating TME to deplete SDS. Since the 1 kDa MWCO membrane shows increased recovery for smaller proteins and does not increase the time required for SDS depletion, it was chosen as the preferred TME membrane for all subsequent experiments.

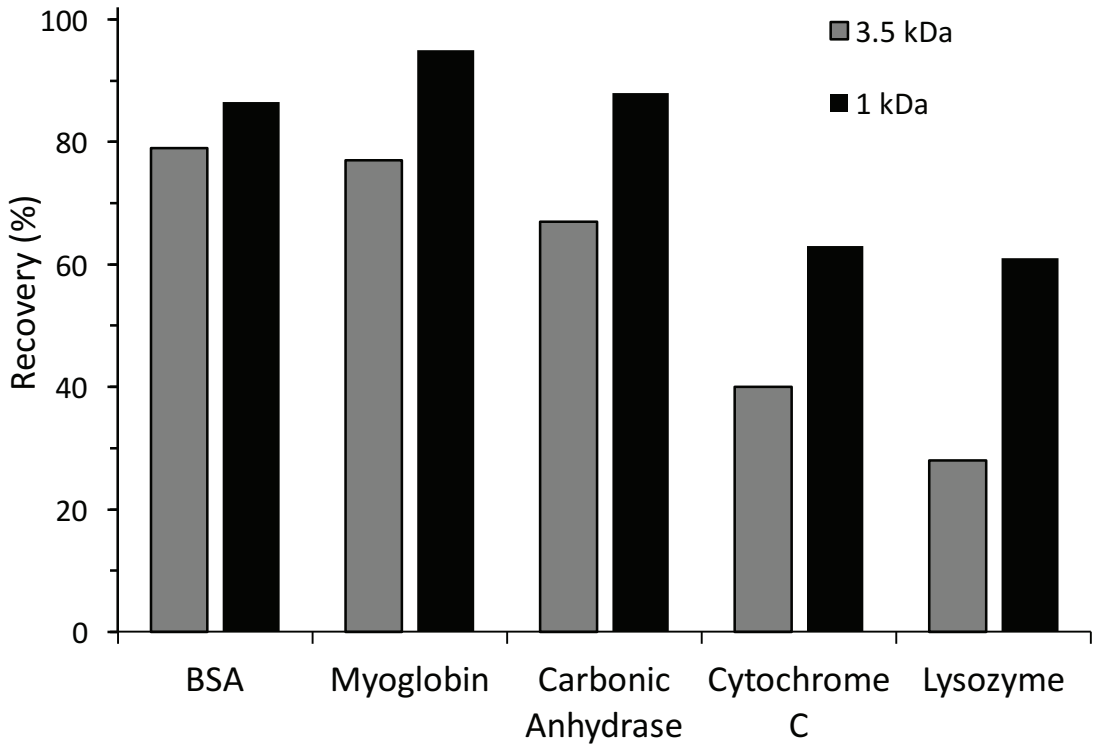

Figure 4-5. Protein recovery of 400 µL of 0.1 g/L cytochrome C and lysozyme when using 3.5 kDa and 1 kDa MWCO dialysis membranes following TME purification.

4.3.2 TME and Temperature

4.3.2.1 SDS Depletion at Decreased Temperature

In Chapter 3, a constant current of 40 mA was observed to deplete SDS to levels compatible with MS, without compromising recovery. When the current was increased to 50 mA, the rate of SDS depletion increased (Figure 3-5). Despite this, TME purification was limited to a constant current of 40 mA because it was noted that increasing to 50 mA resulted in poor protein recovery. It was also noted that at 50 mA, the sample chamber temperature increased to 60 ºC, indicating that an increased temperature had a negative effect on recovery. Thus if temperature could be controlled, then higher cur-

rents could be used without compromising recovery. All experiments presented with TME have been conducted at room temperature. By reducing the temperature, a higher current could be utilized to improve the rate of SDS depletion. However, the effects of temperature on SDS depletion and protein recovery has not yet been studied. There is a potential risk that by decreasing the temperature the time it takes to deplete SDS may increase. This could happen by causing the pores in the dialysis membrane to contract, effectively trapping SDS within the sample chamber, or by simply slowing the rate of ions across the membrane. Using BSA to test the effect of using a reduced temperature on SDS depletion, the TME device, buffer, and sample solution were chilled to 4 ºC prior to running the TME system at 50 mA for one-hour. However, this approach only ensures that the starting temperature is controlled, but does nothing to maintain the temperature while the device is in operation. This was compared to data previously acquired at 50 mA, which started at room temperature (15 ºC). As shown below in Figure 4-6, reducing the temperature to 4 ºC did increase the residual SDS retained in the sample to 3.9 ± 2 ppm. However, this is a marginal increase in comparison to the 1 ± 1 ppm for the data obtained at room temperature, and is identical to the data obtained when operating at 40 mA. Again, it must be noted that the data is presented on a log scale, and these changes in final SDS concentration are negligible considering the initial concentration was 5000 ppm. What is important is to deplete SDS to below 100 ppm. TME surpasses this, depleting SDS to levels below 10 ppm and at reduced temperature SDS can still be depleted within 60 minutes.

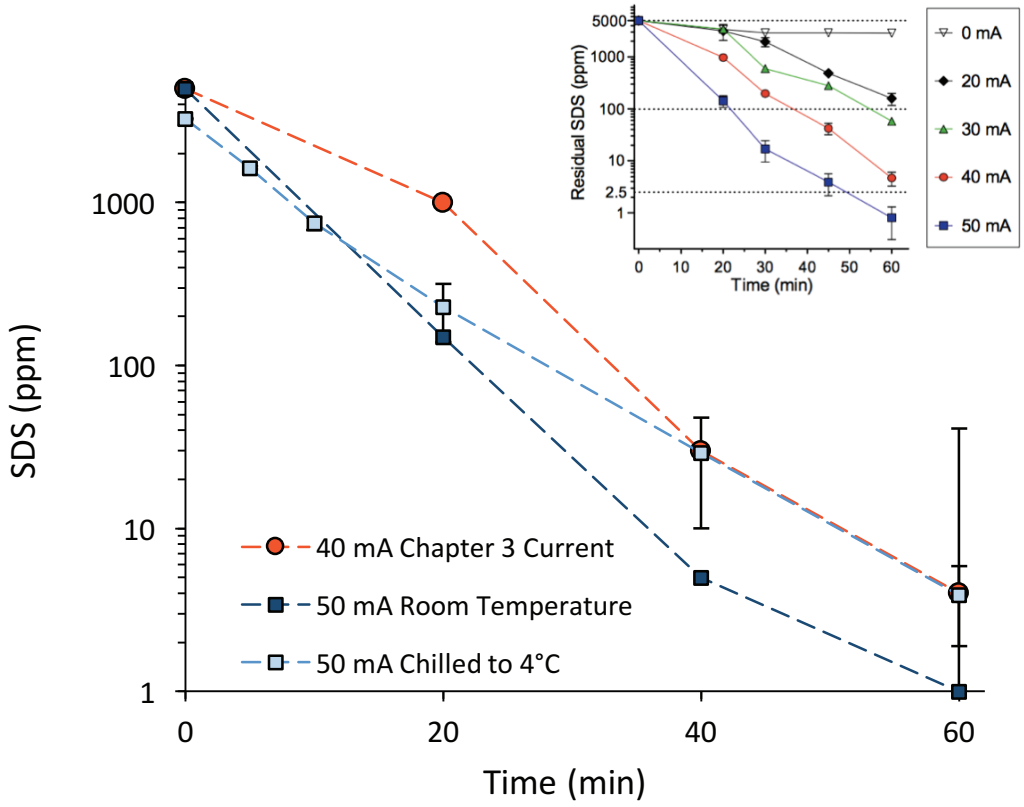

Figure 4-6. A time course experiment of SDS depletion was evaluated to ensure effective SDS depletion despite reduced TME operational temperatures. The apparatus was operating at 50 mA, with the sample chamber temperature initially starting at either room temperature or at 4°C. Inclusion of SDS depletion data from the 40 mA operating conditions in Chapter 3 are also plotted in the figure for comparison purposes. Samples initially contained 40 µg of BSA in 0.5 % SDS. Inset is Figure 3-4 and is shown for comparison purposes.

4.3.2.2 Protein Recovery at Decreased Temperature

The increase in temperature that was observed when operating at 50 mA (*vs* 40 mA) was attributed to Joule heating. High temperatures can cause protein to aggregate, reducing recovery. When starting at room temperature and operating at 50 mA, the tem-

perature rose to 60 °C and caused BSA recovery to drop to 60 % (Figure 3-5). Chilling the device and solutions to 4 °C prior to operation, has the potential to improve recovery. The temperature could still reach high enough temperatures during the one-hour operation to cause protein degradation and reduced recovery. To test this, the system and solutions were chilled to 4 °C and subjected to TME SDS depletion at 50 mA for one hour. As shown in Table 4-1, by simply reducing the initial temperature of the system to 4 ºC, as opposed to starting at room temperature, the final temperature only reached 43 ºC, as opposed to 60 ºC. This likely contributed to an increase in protein recovery from 60 % to 86 ± 17 %. This increase in recovery indicates that temperature potentially plays a very significant role in the success of protein recovery when using TME. Being able to control the temperature of the sample chamber will facilitate the electrical optimization of the TME device, and thus all subsequent experiments begin at 4 ºC.

Table 4-1. Protein recovery of BSA following TME purification at reduced temperature.

Initial Temperature (ºC)	Final Temperature (ºC)	Protein Recovery (%)
15 ºC (Room Temperature)	60 ºC	60 ± 4
4 ºC	43 ºC	87 ± 17

4.3.3 TME and Power

4.3.3.1 SDS Depletion at Increased Power

To test this theory, BSA was depleted of SDS by TME in a series of 12 time course experiments. Each time course experiment was conducted at a higher wattage than the previous and spanned between 8 and 36 W. The first wattage was chosen to be 8

W because previous experiments conducted at 40 mA typically resulted in initial voltages around 200 V across the system. Using the formula $P = I \times V$, 40 mA at these voltages roughly equates to ~ 8 W. Of the 12 time course trials ran, Figure 4-7 shows only a select four (8, 14, 18, and 36 W). The complete data set is presented in Table 4-2, and discussed in Section 4.3.3.2. These four wattages in particular represent four distinct time thresholds for depleting SDS to levels below 10 ppm. Using 8 W, SDS was depleted below 10 ppm in the usual 60 minutes, as expected. Using 14 W, SDS was depleted in 40 minutes. At 18 W, this was reduced to 20 minutes, and finally at 36 W, SDS was depleted within 10 minutes. These milestones occurred when residual SDS values dropped to below 10 ppm SDS in at least two separate trials. These results are quite promising, and can potentially reduce the operation time of TME from 60 minutes to 10 minutes, so long as protein recovery is not compromised.

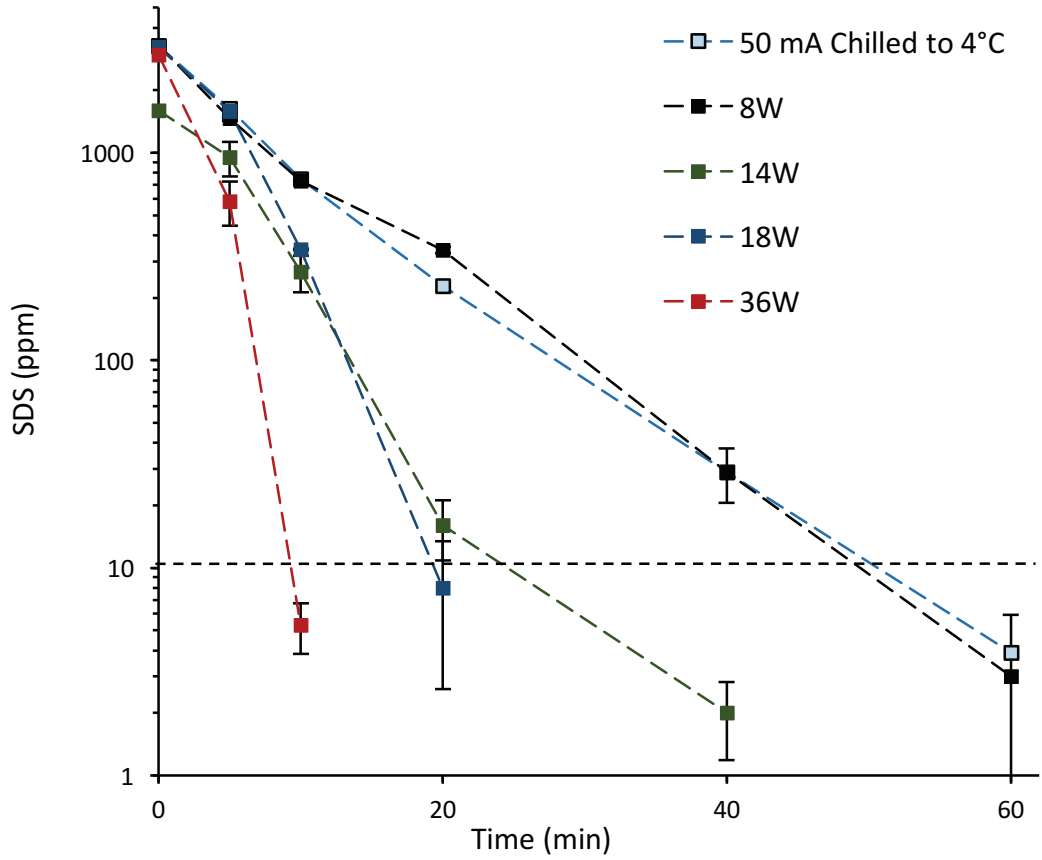

Figure 4-7. Time course experiments of the SDS depletion from 40 µg of BSA in 400 µL of 0.5 % SDS (5000 ppm). Trials when the residual SDS dropped below 10 ppm are shown. n = 8.

4.3.3.2 Protein Recovery at Increased Power

With increased power, SDS depletion is now possible within 10 minutes. Though the effects of Joule heating affecting protein recovery still needs to be investigated. As the applied power increases, the temperature should also increase to a point where protein recovery is compromised. Earlier it was shown that by simply chilling the TME system and solutions to 4 ºC, protein recovery of BSA increased by 25 % when the system was run at 50 mA over a one-hour period. This same approach of chilling the sample prior to

SDS depletion by TME is used here with the expectation that it will aid with protein recovery. The device was also paused as described in Section 4.2.4, which helps control temperature. The Teflon sample chamber of the device heats up quickly because of the high resistance across the membrane and the inability for the heat to dissipate. When the device is paused, the temperature of the sample chamber quickly begins to equilibrate with the buffer chambers, which remain cool throughout the operation of TME (0 min, 4 ºC; 5 min, 5 ºC; 10 min, 8 ºC; 20 min, 11 ºC; 40 min, 16 ºC; 60 min, 19 ºC). At the end of the four SDS depletion time course experiments shown in Figure 4-7, temperature and recovery values were also acquired. Shown in Table 4-2, the experiment run for 60 min at 8 W had the worst protein recovery at 55 ± 20 %. The best protein recovery was seen at 36 W with 88 ± 10 %. This is an interesting result upon first glance, as an increase in wattage would mean an increase in temperature and thus a decrease in recovery. However, the SDS depletion at 36 W occurred in only 10 minutes, thus limiting the temperature increase.

Temperature remains a critical parameter for the operation of TME. A total of 12 time-course data sets were acquired as a function of increased wattages and are shown in Table 4-2. When considering all the data, protein recovery can vary drastically depending on the final temperature. The variation in final temperature shown in Table 4-2 is related to the initial temperature and the length of time the device was paused to collect the time course samples. According to the data in Table 4-2, it appears that when the sample chamber reached a temperature of ~ 60 °C protein recovery was compromised, despite the wattage used. This final temperature appears to correlate directly with the starting temperature of the system. Looking at the pair of data obtained at 14 W, this

point is easier to see. When the temperature started at 4 °C, the final temperature did not exceed 53 °C and good recovery (73 ± 2 %) was attained. When the temperatures started at 11 °C, the temperature rose to 61 °C and protein recovery dropped to 35 ± 7 %. Similar patterns, where increased temperature results in reduced recovery, are also observed at 18 W and 24 W. This suggests that temperature within the sample cell is critical to protein recovery. This may be caused by protein degradation, or the protein may be aggregating and precipitating out of solution. The protein used in these trials, BSA, readily forms hydrophobic aggregates when heated to above 50°C,[364] which do not revert to monomers upon cooling.[365] Thus, it is likely that the proteins are still contained in the sample chamber and can still be recovered. Nonetheless, to improve the recovery of proteins, it is recommended to periodically pause the device, as described in Section 4.2.4. Pausing after every minute of operation for approximately 30 seconds until the sample chamber cools to below 30 ºC, prevents the sample from reaching the 60 ºC critical temperature. Thus, the success of TME purification at higher wattages relies on the ability to maintain the temperature below 60 ºC.

Table 4-2. SDS depletion and protein recovery for BSA with increasing power.

Applied Power (W)	Time for SDS Depletion (min)	Initial Temperature (°C)	Final Temperature (ºC)	Residual SDS (ppm)	Protein Recovery (%)
8 **	60	8	43	3 ± 5	55 ± 20
10	60	18	50	1.0 ± 0.3	33 ± 13
12	40	10	51	4.7 ± 0.1	66 ± 13
14 **	40	4	53	2.4 ± 0.8	73 ± 2
14	40	11	61	3 ± 1	35 ± 7
18 **	20	7	50	1.9 ± 0.1	74 ± 12
18	20	12	69	8 ± 5	57 ± 10
24	20	15	63	3 ± 2	31 ± 12
24	20	15	56	4 ± 2	42 ± 21
32	10	10	52	14 ± 4	76 ± 15
36 **	10	10	58	5 ± 1	88 ± 10
36	10	4	42	7.1 ± 0.1	92 ± 20

* Data is from 12 time course experiments with increasing wattages. n = 4.
** Recovery data is for the same samples shown in Figure 4-7.

4.4 Conclusion

Transmembrane electrophoresis is a powerful device that can be used to deplete SDS from protein samples quickly and efficiently. It consistently depletes SDS to below 10 ppm, which is suitable for subsequent MS analysis. The use of a 1 kDa MWCO membrane improves recovery of proteins less than 30 kDa, without compromising SDS depletion rates. Increasing the power applied to TME to 36 W allows for purification to occur within 10 minutes, without compromising protein recovery. However, when operating at 36 W, the temperature of the device and the samples must be reduced to 4 °C prior to operation to ensure that the final temperature remains low. If the temperature increases to ~ 60 °C, protein losses are observed. Though this is not an ideal situation, a

solution to controlling the temperature while the device is in operation is underway and will be presented in the future. Possible ways to accomplish this will be discussed in Chapter 5: Conclusion and Future Recommendations.

Chapter 5: Conclusion and Future Recommendations

There are many published methods that address the need to remove SDS from protein samples ahead of MS analysis. Such methods include dialysis, column-based separations, filtration, electrophoretic gel-based methods, and precipitation methods. The majority of these methods are only suited towards bottom-up mass based protein identification as they require digestion to recover protein following SDS depletion. Protein precipitation is an exception and can be used with both bottom-up and top-down workflows; however precise pipetting is required to avoid sample loss, with particular care needed at low protein concentrations.

Chapter 2 quantitatively compares eight commonly used SDS depletion methods in literature (FASP, acetone precipitation, TCA precipitation, KCl precipitation, Pierce detergent removal spin columns for peptide and for protein, strong cation exchange, and SDS-PAGE with in-gel digestion). They were assessed using BSA and *E. coli* at 0.1 and 1.0 g/L. Though five out of the eight protocols removed SDS to levels below 10 ppm, FASP removed the greatest amount of SDS. SDS depletion is not the only factor that needs to be considered when utilizing these methods; protein recovery is just as important. When looking at recovery values, acetone precipitation had the highest average recovery. This does not necessarily translate directly into increased protein identifications, so FASP and acetone precipitation were chosen to evaluate protein *vs* purity recovery on bottom-up proteome analysis. Using GELFrEE to divide the *E. coli* proteome into a high and low fraction, acetone precipitation identified more peptides and proteins than those identified with FASP, with minimal overlap of identifications. Analysis of cellular components, hydrophobicity, isoelectric point, peptide mass, and the number of missed

cleavages did not show any specific bias that separates the type of protein identified by either method. With several choices available for SDS depletion, acetone precipitation comes out as a preferred choice that enables high protein recovery and exceptional SDS depletion. Despite the high recovery attainable by acetone precipitations, it does require precise pipetting to avoid sample loss, which can be particularly difficult at low protein concentration where visualization of the protein pellet is difficult. Thus, the proteomics community is reluctant to incorporate it as a reliable, easy to use approach.

To address the fact that there is no 'universal' SDS depletion method, Chapter 3 introduces transmembrane electrophoresis (TME), a process that combines the principles of dialysis with the power of a potential gradient. TME has the potential to be widely incorporated into proteomic workflows, particularly those that employ top-down MS-base protein identification protocols. It is a simple to use device that removes SDS to levels below 10 ppm at the push of a button. TME consistently retains greater than 90 % of protein, for both standard test proteins (BSA and myoglobin) and for whole cell *E. coli* proteomes. TME can be used in both bottom-up and top-down mass based protein identification strategies and is particularly suited to link GELFrEE separation to MS analysis in the top-down workflow. The design of TME captures all proteins within the center sample chamber, regardless of solubility. This is particularly advantageous for membrane protein analysis, where their hydrophobic nature compromises recovery when using other SDS depletion strategies. By utilizing a cold formic acid wash, any precipitated hydrophobic proteins can be quickly recovered. Subsequent analysis of a membrane enriched *E. coli* proteome revealed high protein recovery with more than half being membrane or membrane-associated. Of these membrane proteins, 96 % contained amino acids that ei-

ther traversed the membrane or were anchored / embedded in the membrane. Even highly hydrophobic proteins, as determined by GRAVY scores above +1, were recovered from the sample chamber and identified. A significant advantage of TME over other methods is its amenability to top-down protein identification workflows. As demonstrated in Chapter 3, intact membrane proteins were identified with distinct charge state envelopes and high signal to noise ratios. Deconvolution of the spectra identified several proteins with no SDS adducts or formylation events (from the use of formic acid). Though true top-down proteome analysis entails tandem MS, these results indicate that TME has the potential to be used in these MS-based protein identification strategies.

Though the original one-hour time for SDS depletion is better than most other SDS depletion methods, the final goal in the development of the TME device is to incorporate it into an in-line system. To work towards this, Chapter 4 examines improvements to the design that resulted in decreasing the time required to deplete SDS. Changing the pore size of the MWCO dialysis membranes used to partition SDS and protein did not significantly change the time required to deplete SDS. However, the use of a smaller pore size did improve recovery of smaller (< 30 kDa) proteins and was chosen as the preferred membrane size for subsequent experiments. Changing the operation of the system to a high constant power (wattage) enabled SDS depletion to improve to ten minutes. The protein recovery showed variable results, which correlated with an increase in temperature to 60 °C. Though other parameters could influence the effectiveness of the TME device (*e.g.* pH, sample volume, buffer volume, and sample additives) and warrant investigation, temperature appears to be critical. So long as temperature can be controlled, de-

pletion of SDS within ten minutes provides reliable and consistent results for SDS depletion and high protein recovery.

Currently, temperature control is achieved by periodically pausing the system to allow the inner sample chamber solution to drop to below 30 ºC. This pause is quick, taking approximately 30 seconds, but is certainly not an ideal solution to maintaining or monitoring the temperature. The development of an automatic temperature control system is therefore essential to the progression of TME to an inline device. This could be accomplished by simply changing in the physical design of the device. During the operation of TME, the outside buffer chambers remain chilled (< 10 °C). By making the sample chamber wider and thinner, the sample should have a larger surface area to interact with the outer buffer solution, allowing for greater heat dissipation. Once the temperature can be controlled, further improvement to the rate of SDS depletion can be achieved.

The physical design of the system comprises of three distinct chambers that are clamped together. Thus, for every change to the physical design, particularly of the sample chamber, three distinct pieces require fabrication. A new design, where one box can be divided by a sample cassette, would allow for easy testing of a variety of cassettes. These cassettes could test different thicknesses, have larger or smaller surface area exposed to the buffer chambers, or simply contain more sample chambers to improve the number of samples that can be processed at a time. Once the effects these parameters have on TME have been explored and understood, it can be effectively incorporated into an inline device directly linked to a mass spectrometer.

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FILE S6: PROTEIN LIST – HIGH MW, FASP II {FASPII _HIGHMASS_PROTEIN.XLS}

FILE S7: PEPTIDE LIST – LOW MW, FASP II {FASPII _LOWMASS_PEPTIDE.XLS}

FILE S8: PROTEIN LIST – LOW MW, FASP II {FASPII _LOWMASS_PROTEIN.XLS}

FILE S9: EXCEL FILE OF IDENTIFIED WHOLE CELL *E. COLI* PROTEINS;

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