THE DEVELOPMENT OF SAMPLE PREPARATION METHODS INVOLVING DETERGENTS FOR ANALYSIS OF MEMBRANE PROTEINS BY MASS SPECTROMETRY

by

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ABSTRACT

Appropriate solubilization of membrane proteins can be achieved by incorporating detergents such as sodium dodecyl sulfate (SDS). Unfortunately, SDS interferes with liquid chromatography (LC) and mass spectrometry (MS). This thesis presents "MS-friendly' alternatives to solubilize membrane proteins and provides an evaluation of current protocols for SDS removal.

Considering the limitation of SDS in a proteome analysis workflow, fluorinated surfactants have previously been proposed. Our results showed that APFO similar proteome solubilization to that of SDS. Unfortunately, APFO was only marginally more tolerable to LC and MS than SDS. Nonetheless, an important advantage of using APFO is that it can be easily removed from the sample by evaporation.

The efficiency of precipitation protocols was explored. Our results demonstrate that high recovery is possible. Quantitative re-solubilization of membrane proteins following precipitation was made possible through addition of 80% formic acid. This solvent system may present a promising pathway for top-down MS analysis.

List of Abbreviations and Symbols Used

2D PAGE	Two-dimensional polyacrylamide gel electrophoresis
1D	One dimensional
2D	Two dimensional
ACN	Acetonitrile
APFO	Ammonium perfluorooctanoate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CE	Capillary electrophoresis
CID	Collision-induced dissociation
CMC	Critical micelle concentration
CMW	Chloroform/ methanol/ water
DTT	Dithiothreitol
ECD	Electron capture dissociation
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
ESI-MS	Electrospray ionization-mass spectrometry
ETD	Electron transfer dissociation
FTMS	Fourier transform mass spectrometry
GELFrEE	GEL-eluted liquid fraction entrapment electrophoresis
HPLC	High performance liquid chromatography
IEC	Ion-exchange chromatography

IEF	Isoelectric focusing
LC	Liquid chromatography
LC/ MS/ MS	Liquid chromatography/ tandem mass spectrometry
LC/MS	Liquid chromatography/ mass spectrometry
m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption/ ionization
MEKC	Micellar electrokinetic chromatography
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MudPIT	Multidimensional protein identification technology
NCBI	National center for biotechnology information
PBS	Phosphate buffered saline
PFO/PAGE	Perfluorooctanoic acid/ polyacrylamide gel electrophoresis
PFOA	Perfluorooctanoic acid
PPS	Sodium 3-(4-(1,1-bis(hexyloxy)ethyl)pyridinium-1-yl)propane-1-sulfonate
PSM	Peptide spectrum match
PTMs	Post translational modifications
QTOF	Quadrupole time-of-flight
RPLC	Reversed phase liquid chromatography
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEC-CE	Size-exclusion chromatography capillary electrophoresis
SEC-RPLC	Size-exclusion reversed-phased liquid chromatography
sIEF	Solution isoelectric focusing
TFA	Trifluoroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet

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Chapter 1
Introduction

1. 1 Overview

The high throughput study of the proteome (*i.e.* proteomics) can perhaps trace its beginnings to the mid 1970s, with the development of two-dimensional polyacrylamide gel electrophoresis (2D PAGE), thereby permitting simultaneous separation and visualization of thousands of proteins in a single experiment. [1] Since this time, numerous technological advances have translated into an exponential level of growth in the field. [2] Proteomics can be defined as the qualitative and quantitative characterization of the full complement of proteins expressed by a cell or tissue under a given set of conditions. [3] Though modern strategies for proteome analysis have matured considerably since its most early beginnings, [2] numerous analytical challenges remain. Mass spectrometry (MS) is now the instrument of choice for proteome characterization. In fact, the explosion in MS growth seen in recent years, including the development of the QTOF (quadrupole time-of-flight), [4] QTRAP, [5] Orbitrap [6] and high field FTICR (Fourier transform ion cyclotron resonance) [7] is a direct response to the high demands brought on by proteomics researchers (*i.e.* higher sensitivity, resolution and scan speed). [8-10]

Regardless of the power of the MS platform, it must be noted that the proper preparation of a proteome sample, ahead of analysis, ultimately dictates the success of the analysis. It is these critical front-end technologies which continue to present significant limitations in the field and are therefore the focus of this thesis. Concerning membrane proteins in particular, special attention to sample preparation must be taken in order for MS characterization to be successful. These proteins are much more hydrophobic, and are

therefore poorly soluble in water, meaning that solubilization additives are generally required to assist the dissolution of membrane proteins. [11] Unfortunately, additives such as ionic surfactants are, for the most part, incompatible with mass spectrometry, as well as with liquid chromatography (LC), which is an integral component of the proteome detection platform (*i.e.* LC/MS). In this introductory chapter, the motivation and challenges of membrane proteome analysis by MS will be described. An overview of current methods for membrane proteome analysis is presented, highlighting the importance of surfactants for protein solubilization as well as the separation technologies for fractionation of the proteome ahead of MS.

1.2 Membrane proteins: why are they difficult to study?

Membrane proteome analysis is potentially a powerful strategy for identification of specific and novel biomarkers that may be utilized for prognosis and monitoring of several diseases. Approximately 20% of all proteins encoded by the mammalian genome can be classified as membrane proteins, [12] yet membrane proteins currently represent more than two-thirds of the protein targets for potential drugs. [13] Understanding changes induced within the membrane proteome across healthy and diseased states is therefore critical as a means of uncovering or validating novel protein biomarkers.

Membrane proteins are associated with the cell lipid bilayer, being either integral membrane proteins (permanently bonded to the membrane) or peripheral membrane proteins (temporarily bonded to either the membrane or to integral membrane proteins). Integral membrane proteins, or transmembrane proteins, have portions of the protein penetrating through the lipid bilayer, typically in the form of α-helices or β-sheets (see Figure 1.1). [14] Analysis of α-helix-containing transmembrane domains present a major

challenge to proteomics characterization mainly due to their low abundance and hydrophobicity. As a result, the analysis of membrane proteins, especially integral membrane proteins with multiple alpha helical transmembrane domains, is generally more difficult to conduct using MS due to their high level of hydrophobicity. For most membrane proteins that act as channels across the lipid bilayer, a β-sheet structure composed of polar amino acid side chains line an inward-facing aqueous channel, while non-polar amino acids interface with the lipid bilayer. [14, 15] Since β-sheets do not have consecutive non-polar amino acids, they are considerably more hydrophilic than α-helical integral proteins. Thus, the overall hydropathy of the β-sheets is similar to that of soluble proteins, and those proteins are therefore not as difficult to analyze.

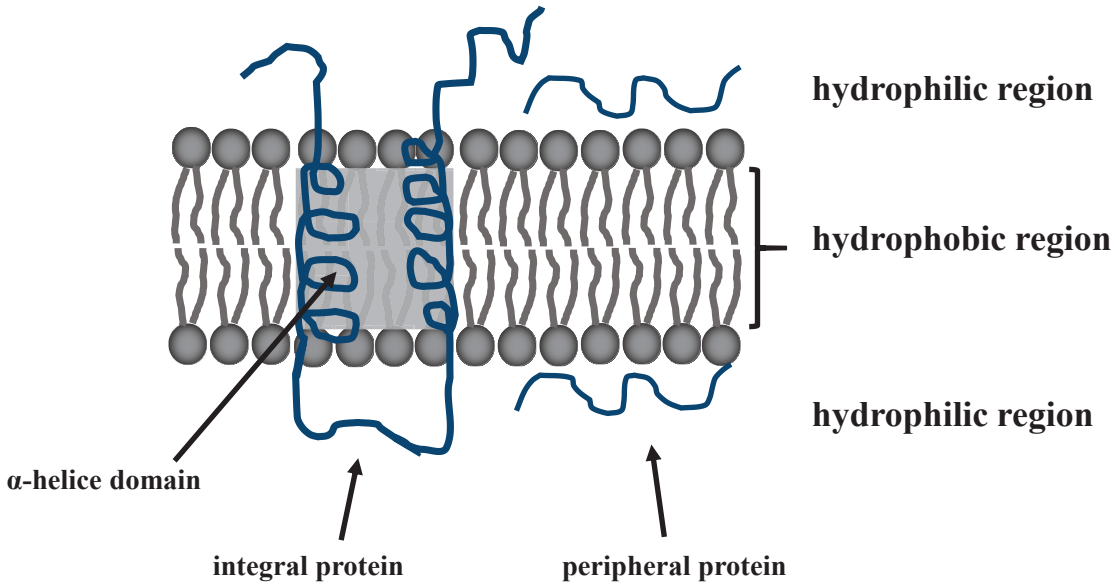

Figure 1.1 The association of membrane proteins (blue) with the cell lipid bilayer (gray). Integral proteins are permanently bonded to the membrane (with protein domains in the hydrophobic and hydrophilic region) while peripheral proteins are usually temporarily bonded to the membrane or to integral membrane proteins (often at a hydrophilic region).

The characterization of membrane proteins by mass spectrometry is significantly more difficult than for water-soluble proteins. Typically, isolation of membrane proteins

from the membrane is achieved only in a membrane-like environment that is most easily mimicked, *in vitro*, by detergents (or surfactants). [16] The most efficient method to solubilize membrane proteins involves the use of an anionic surfactant like sodium dodecyl sulfate (SDS). [17] This detergent is commonly used for mass-based separation through SDS-polyacrylamide gel electrophoresis (SDS-PAGE). As shown in Figure 1.2, SDS works by disrupting non-covalent bonds in the proteins, causing the biopolymer to denature. While the tightly bound SDS-protein complex ensures solubilization of the protein, these interactions are also directly responsible for incompatibility issues with downstream proteome analysis procedures. For example, SDS reduces the activity of proteolytic enzymes (*e.g.* trypsin), which are an integral part of the proteomics workflow. [18] These interactions also disrupt chromatographic separation (ion exchange/reversed-phase) and further suppress ionization of the peptides or proteins by matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI). [19-21] The use of detergents therefore presents a dilemma in the field of proteomics; while desired for protein solubilization, they are incompatible with MS.

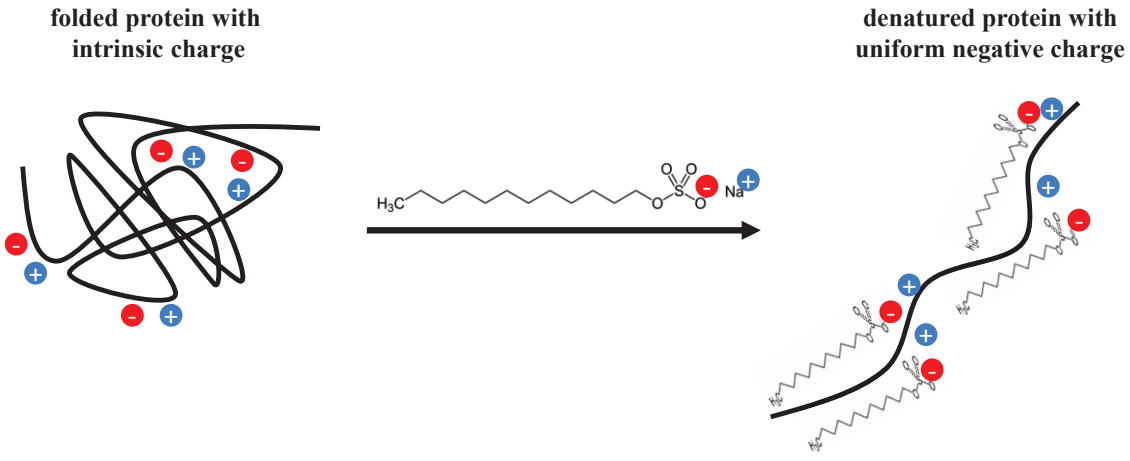

Figure 1.2 An illustration of the effect of SDS on the conformation and charge of a protein. SDS disrupts non-covalent bonds in the protein, causing the molecule to denature.

Considering the importance of ionic surfactants for protein solubilization, it would be desirable to allow such compounds to become part of the proteomics workflow, at least in the early stages (*e.g.* proteome extraction from the cell and initial solubilization). In doing so, one would therefore require a method to either reduce the concentration of SDS prior to the latter stages of the workflow where it would otherwise interfere (*e.g.* during LC separation and MS detection). Alternatively, a solubilizing additive that is compatible with all aspects of the proteomics workflow can be incorporated, in place of SDS. The strategy of removing SDS may be addressed by introducing a cleanup step prior to protein digestion (typically enzymatically, to form peptide fragments for subsequent MS analysis). For example, proteins may be precipitated, leaving the SDS in solution, and following extraction, re-solubilizing the protein in a MS-compatible solvent (see Chapter 3). ESI mass spectra of membrane proteins have been obtained in organic solvent systems such as chloroform/methanol/water/formic acid, [22] chloroform/methanol/water, [23] formic acid/methanol/water [24] and formic acid/isopropanol. [25] However, these methods vary in terms of their ability to completely solubilize the membrane proteins, with protein loss being observed prior to mass spectrometry analysis. In Chapter 3, an evaluation of common precipitation techniques for SDS removal is reported, with emphasise on the recovery of membrane proteins through precipitation, followed by resolubilization of the sample.

1.3 General properties of surfactant and protein-surfactant interactions

Surfactants are amphiphilic molecules, having a polar group as well as a hydrophobic moiety (*e.g.* alkyl chain). Surfactants usually adopt a specific macromolecular structure in aqueous solution, known as micelles, with a generally hydrophobic interior and a hydrophilic water-exposed exterior, as shown in Figure 1.3. The critical concentration of

surfactant to form these micelles is called the critical micelle concentration (CMC). CMC values are very important as proteins interact differently with the micellar or monomeric surfactant. Two important aspects of the CMC will be discussed. First, the CMC is very sensitive to ionic strength, and therefore the CMC should be determined for given buffer conditions (*e.g.* the CMC of SDS in water is 7-8 mM while in PBS, or phosphate buffered saline, it drops to near 0.8 to 1 mM); [26] Second, the formation of micelles is affected by the presence of proteins, since these polypeptides sequester surfactant molecules and then reduce the concentration of free monomeric surfactant. [26] In other words, high concentration of proteins decreases the CMC formation. However, the situation is more complex for membrane proteins (*i.e.* mainly hydrophobic proteins) in that a continuous amphiphilic region is required to shield the hydrophobic transmembrane regions of the protein. [16] Also, membrane proteins tend to aggregate below the CMC of the surfactant, and therefore require a high concentration of surfactant to maintain protein solubility.

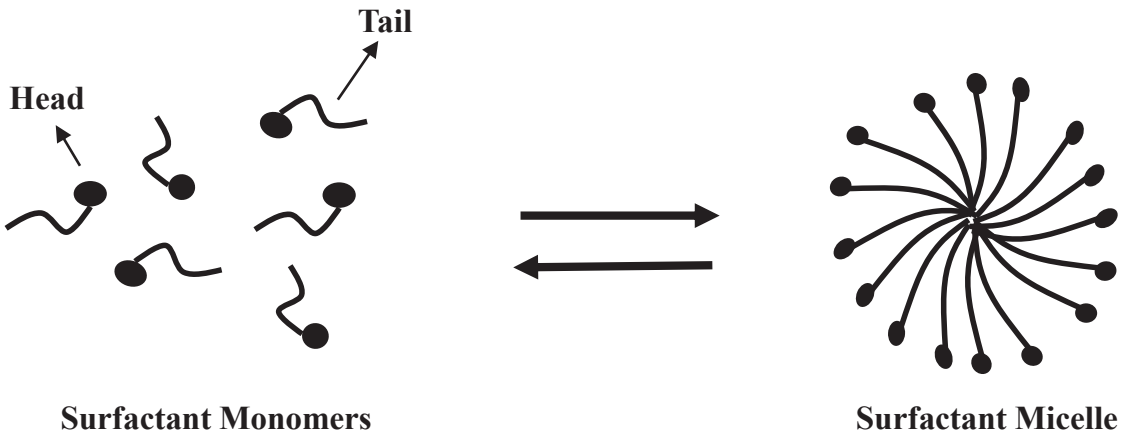

Figure 1.3 An illustration of the reversible monomer-micelle structure. These amphiphilic molecules are composed of a hydrophilic or polar moiety, often referred to as the head (shown as a black circle) as well as a hydrophobic or nonpolar moiety known as the tail (shown as curve lines). When surfactants molecules are dissolved in water at concentrations above the CMC, they form aggregates known as micelles.

Protein-surfactant binding (ionic surfactants) proceeds in several steps, the process of which is considered quite complex. The first step involves monomers binding *via* electrostatic and hydrophobic interactions. [27] Anionic surfactants (*e.g.* SDS) interact with cationic side chains including lysine, arginine and/or histidine, while cationic surfactant binds anionic side chains such as glutamic acid and aspartic acid. For both classes of compounds (cationic/anionic), the alkyl chains of the surfactant will bind to nearby hydrophobic patches of the protein. [27] In the presence of a higher concentration of surfactant, the initial binding sites become saturated, and so the binding of more surfactant might lead to clusters that start to unfold the protein. These clusters play an important role in the properties of protein-surfactant complex at low surfactant/ protein ratio. At a relatively low surfactant concentration, the formation of shared micelles is driven by small protein complexes. [27] Higher surfactant concentrations, but still sub-CMC, provide sufficient surfactant to allow proteins to form a cluster on their own, and therefore when the surfactant concentrations approaches its CMC, the rate of unfolding tends to level off. [27] The longer the chain length, the greater the degree of binding, and therefore the greater the protein stabilization. [28]

1.4 The use of surfactant in proteomics

Surfactants, being used to solubilize a wide range of proteins, often interfere with LC separation and/or cause MS signal suppression. With reversed-phase liquid chromatography (RPLC), protein or peptide separation is affected by small amounts of SDS (see Chapter 2 for further details). As little as 0.01% SDS can significantly deteriorate the resolution and alter the retention time of proteins and peptides in RPLC. [29] The preferred surfactant for proteome solubilization, SDS, may also drastically impact the MS

electrospray ionization process. [29] While low concentrations of SDS have been shown to enhance the MALDI ionization process, [30] higher concentrations of this anionic surfactant also significantly decreases MALDI ionization process. For ESI signal intensity, the matrix effect caused by SDS is observed at very low concentration. [29] Using ESI, Botelho *et al.* [29] demonstrate that, at or above 0.01% SDS, the identification of proteins was significantly compromised in a typical proteome experiment in which 1 µg total protein is separated on a capillary column with nanospray ionization. This was attributed to poor ionization efficiency (see Figure 1.4), together with disrupting the retention of peptides during reversed-phase liquid chromatography. [29]

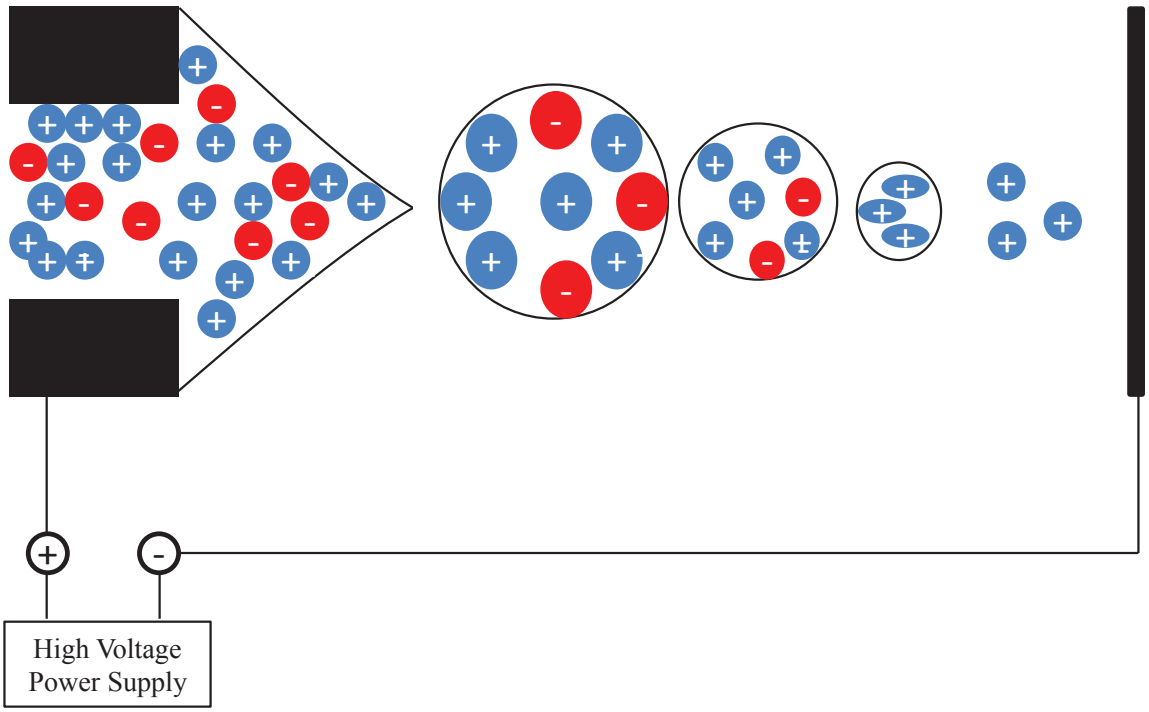

Figure 1.4 Illustrates the Taylor cone and charged droplet formation during electrospray ionization (positive mode). A low concentration of highly surface active anionic surfactants (negative symbol) may reduce the efficiency of the electrospray process due to their preference to partition to the surface, which could affect the Taylor cone formation.

The mechanism of signal suppression by anionic surfactants in electrospray ionization-mass spectrometry (ESI-MS) has been the subject of several studies. [29, 31] Figure 1.4 shows the general process of electrospray ionization. High voltage, applied to the capillary tip, produces charged droplets that are attracted toward the counter electrode, that being the entrance of the MS instrument. Following initial formation, the charged droplets undergo partial desolvation until the Rayleigh limit is reached. When the Coulombic repulsion of the ions in the droplet is strong enough to overcome the liquid surface tension, the droplet is disrupted. The resulting droplets are smaller and enriched in charge compared to the parent droplets. However, at low concentrations of anionic surfactants like SDS, the analyte ion signals are completely destroyed. [29] Rundlett *et al.* have demonstrated two important effects of SDS for the suppression mechanism on ESI-MS in the positive mode. [31] The first effect is related to the reduction in the amount of solution that can be sprayed by the ESI interface. Surfactant ions (represented by negative charge in Figure 1.4) at the ESI interface often destabilize the Taylor cone formation, and therefore there is a reduction of spray efficiency. The second effect is interference with the transfer of cations ions from the droplets to the gas phase due to Coulombic attractions between oppositely charged analyte and surfactant in the droplets produced by the electrospray mechanism. These concepts suggest that the ionization suppression mechanism of surfactants is mainly due to a charge-charge interaction.

Beyond instrumental concerns, yet another disadvantage of using SDS in the proteomics workflow is that it affects enzyme digestion efficiency. This occurs at concentrations similar to the CMC of the surfactant (*e.g.* above 0.2% SDS). [18] Protein digestion with enzymes such as trypsin is an integral aspect of the proteomics workflow,

as peptide fragments are sequenced by tandem mass spectrometry (see Section 1.6). SDS inhibits the action of enzymes by denaturing the enzyme itself, which reduces its catalytic activity. To overcome this, proteins have been dissolved at high levels of SDS (*e.g.* ~1%), then diluted with water prior to addition of the digestion enzyme (*e.g.* to ~0.1%), which is sufficiently low to be tolerated for digestion. [32] However, this method is not reliable, particularly for membrane proteins, as reducing the SDS/protein ratio (1.4 to 1 by mass) [33] is ultimately not favored to maintain protein solubility.

The benefit of using surfactants for protein solubilization as well as for separation (*e.g.* SDS-PAGE [1] or GELFrEE [34]) may still be gained if the detergent is removed prior to LC separation or MS ionization. Several efforts have been made to effectively remove surfactants from the sample. Methods for detergent removal include protein precipitation, dialysis, or column based approaches (*e.g.* ion exchange, gel filtration). [35-37] The SDS removal efficiency, protein yield and the reproducibility of the method vary greatly according to the method utilized. For instance, even if a large proportion of SDS is removed by one of the techniques listed above, the residual presence of a small amount of this anionic detergent, being strongly associated with cationic side chains of amino acids, can ultimately deter LC/MS analysis. A universal approach to completely remove all surfactant from protein samples (while maintaining high protein recovery) is unknown, which has led to several research groups adopting their own strategy for detergent removal prior to MS analysis. Chapter 3 explores the efficiency of common SDS removal methods (protein precipitation with acetone or with chloroform/methanol/water) for analysis of membrane proteins through MS.

1.5 Alternatives for solubilization of proteins

The discovery of alternative surfactants for solubilizing membrane proteins, which are either directly compatible with MS, or can easily be removed prior to MS, has been a target of intense research in the proteomics field. [18] Low-boiling point surfactants have been considered a potential alternative mainly due to their possible MS compatibility. [38] Fluorinated surfactants are among a class of surfactant that has low boiling point and are both hydrophobic and lipophobic, being extremely effective to decrease water surface tension. [39] Perfluorooctanoic acid (PFOA) is a perfluorinated carboxylic acid that was originally utilized to prepare fluorinated polymers such as polytetrafluoroethylene, PTFE, (Teflon). [40] The attraction of PFOA to proteomics experiments began when various laboratories utilized this perfluorinated surfactant to solubilize membrane proteins. [38, 39, 41] Chapter 2 gives a detailed investigation of the tolerance of ammonium perfluorooctanoate towards LC, MS and LC/MS analysis.

Another surfactant alternative is the use of phase-transfer surfactants such as sodium deoxycholate (SDC). Such a surfactant can easily be removed prior to mass spectrometry by adding organic solvents to an acidified solution, thereby extracting the surfactant. [17, 43] The use of these surfactants has additional benefits over SDS in that it can enhance the activity of trypsin. [17] Masuda *et al.* [17] have demonstrated that the activity of trypsin may be enhanced nearly 5-fold in the presence of 1% SDC. Lin *et al.* [42] showed compatibility of SDC with tryptic digestion at concentrations up to 5%. The reason for high compatibility with trypsin is based on the fact that this system is a mimic of the alimentary canal in which bile salts such as SDC are secreted together with trypsin. [17] However, the

main shortcoming of SDC is that its ability to lyse membranes and extract the membrane proteins is considerably less effective than that of SDS. [44]

Membrane protein solubilization may also be facilitated without the use of surfactants. Perhaps, the most used method of protein re-solubilization is executed by chaotropes (*i.e.* amide and urea families). These chaotropes break the inter- and intra-molecular non-covalent interactions in the sample (*e.g.* hydrogen bonds, hydrophobic interactions), facilitating protein unfolding. [45, 46] Among the chaotropes, high concentrations of urea have been widely used to re-solubilize proteins. Alternatively, organic acids (*e.g.* 80-90% formic acid) [32] have been utilized for membrane disruption and solubilization of membrane proteins. Like urea, the major advantage of using formic acid as a solubilizing agent is that it is compatible with downstream micro/nanoscale reversed-phase liquid chromatography and electrospray ionization tandem mass spectrometry analysis, reducing sample handling and potential loss.

1.6 Proteome analysis: bottom-up *vs* top-down proteomics

Given the many technical options available for proteome analysis, several general strategies of protein identification have emerged. Bottom-up and top-down experiments are considered the two major approaches. For bottom-up experiments, MS identifies proteins following enzymatic or chemical digestion of the sample, resulting in the formation of peptide fragments, [47, 48] while top-down proteomics is the direct analysis of protein at the intact level. [49, 50]

The bottom-up approach is the most popular method when dealing with large-scale analyses of highly complex samples. Bottom-up proteomics is an approach in which peptide masses are sequenced using tandem mass spectrometry (MS/MS) and are used to

identify corresponding proteins. A protein is first enzymatically digested, which produces a set of peptides with predictable masses. Trypsin is the most common enzyme employed in this strategy. Trypsin is an endoprotease which cleaves the amide bond on the carboxyl side of lysine and arginine residues. The use of trypsin has become popular due to the incorporation of at least one basic amino acid residue into the created peptides, which guarantees a location for positive MS ionization through protonation.

The identification of proteins based on peptide sequencing using tandem mass spectrometry is typically performed through collision-induced dissociation (CID). This process involves the application of energy to promote collisions with an inert gas, often helium, within a collision chamber of the mass spectrometer. The experimental MS/MS fragmentation data can be compared against the predicted pattern of the peptides under investigation. [51] MS/MS identification using a method known as triple play was used to generate Figure 2.10, shown in Chapter 2. Triple play refers to the three step process of (i) ion selection, (ii) charge state determination and (iii) tandem MS fragmentation. However, peptide sequencing relies of the knowledge the amino acid sequence of all proteins expressed in a proteome. Thus, genomic databases such as NCBI (National Center for Biotechnology Information) [52] are extremely important for protein identification. Also, a quick analysis of several MS/MS peptide spectra can be performed by using search engines such as MASCOT [53] and SEQUEST. [54] These algorithms match the experimentally collected fragmentation spectra to computationally generated spectra of potential peptide matches from proteomics databases.

Compared to top-down experiments, bottom-up proteomics has the advantage of analyzing smaller protein segments (*i.e.* peptides) and can therefore be performed on a low

resolution mass spectrometer. However, bottom-up proteomics brings with it the undesirable potential for limited protein sequence coverage and a poor ability to detect all post-translational modifications (PTMs). These drawbacks are mainly due to the small and variable fraction of peptides detected from the enzymatic digestion. Furthermore, portions of proteins (on which PTMs may be located) may go undetected since the typical peptides generated from trypsin usually contain only 5 to 20 amino acids. [55]

In contrast to bottom-up MS, top-down MS analyzes intact proteins without proteolytic digestion, which preserves the labile structural characteristics. Some of the benefits of the top-down approach include higher sequence coverage of target proteins [56] and better characterization of the post-translational modifications. [57] As higher sequence coverage is generally viewed, top-down proteomics reduces the ambiguities of protein identification. [58] Another advantage of the top-down experiment is the improvement in protein quantification [59] where protein abundances are measured directly instead of using the abundances of peptides. However, there are several technological limitations to the top-down method, which keeps it from widespread use. Perhaps the most difficult aspects of intact protein analysis are the lower sensitivity achieved from analysis of multiply charged ions, together with the difficulty associated with the interpretation of fragmentation spectra. Thus, top-down analysis has only recently progressed following developments of high resolution MS instrumentation (*e.g.* FTICR [7] and the LTQ-Orbitrap [6]), as well as improved strategies for fragmentation of large molecules, namely electron capture dissociation (ECD) [123] and electron transfer dissociation (ETD). [10] In addition, software platforms for MS/MS spectral interpretation have allowed for automated deciphering of the complex MS/MS spectra patterns resulting from fragmentation of large, intact

biomolecules. [60, 61] The analysis of intact proteins rather than peptide segments, redirects all front-end manipulations towards larger biomolecules. As discussed in the following section, the separation of intact proteins prior to MS is far more complicated than the separation of peptides.

1.7 Proteomic workflows

Proteome prefractionation may be considered a formidable tool to find low-abundance membrane proteins. Indeed, proteome prefractionation plays a crucial role in proteome analysis strategies. For example, prefractionation may dramatically increase the number of components identified. [124] Prefractionation of samples reduces the complexity of samples by fractionating the complete proteome. Thus, all sample components are retained across the collected fractions. [62] To avoid ambiguity, Doucette *et al.* [63] have defined prefractionation as any form of separation of a complex proteome mixture conducted at the level of intact proteins (*e.g.* sIEF [solution isoelectric focusing], [64] gel electrophoresis, [1] liquid chromatography and liquid electrophoresis [34] methods), prior to subsequent peptide separation analysis (*e.g.* shotgun approaches such as 1D or 2D PAGE LC/MS/MS and multidimensional protein identification technology, MudPIT).

Current methods of pre-fractionation may be divided into two groups: gel-based (*e.g.* 1D or 2D gel electrophoresis) and gel free (solution based) methods (*e.g.* 2D-liquid chromatography, sIEF, GELFrEE). In terms of resolution, the leading technology for prefractionation of intact proteins is two-dimensional polyacrylamide gel electrophoresis (2D PAGE). This pre-fractionation method is capable of resolving more than one thousand intact proteins. [1] Although the 2D PAGE system can isolate a variety of proteins, the proteins visualized on a 2D gel represents “only” a small portion of all possible proteins

expressed by the cell. [65] This limitation is due to the detection of multiply modified forms of a limited numbers of proteins; moreover, low abundant proteins are rarely observed in the 2D PAGE system. [65] Another limitation of 2D gels is that highly hydrophobic membrane proteins are usually undetected. The under-representation of highly hydrophobic proteins may be attributed to factors such as their low solubility and their tendency to aggregate and precipitate in aqueous media. Also, many membrane proteins possess basic pIs (isoelectric points) and/or are expressed in low copy numbers. [65] Certain loss of these membrane proteins may also be related to the fact that they do not elute during the transfer steps from the first dimension (isoelectric focusing, IEF) to second dimension (SDS-PAGE). In the proteomics experiment, the proteins visualized by 1D or 2D PAGE are most often subjected to in-gel trypsin digestion [66] being followed by extraction of the resulting peptides from the gel. This procedure is quite laborious and can result in inefficient extraction of peptides from the gel. The extraction of intact proteins from a gel using solvents is inefficient, and thus 1D and 2D gels are not a convenient separation platform for top-down proteomics. Other modes of proteome fractionation that operate in solution may present a powerful alternative for gel-based fractionations.

During the last decade, there has been a great interest in developing gel-free systems for protein analysis because of their potential for automation through direct coupling to MS. Multiple combinations of HPLC, sIEF, and capillary electrophoresis (CE), for instance, provide various options to develop high-resolution orthogonal 2D liquid-based strategies for the separation of complex mixtures of proteins. Such strategies include size-exclusion chromatography (SEC)-CE or SEC-RPLC. [67, 68] Feng *et al.* [69] purified proteins by using ion-exchange chromatography (IEC) followed on-line eight channel parallel

RPLC-ESI-MS system. [62] Although these liquid chromatography systems may be executed for protein separations (often with poor intact protein recovery, however), they are also excellent for separation of peptides where shotgun proteomics can be performed with an automated system for proteome analysis.

In contrast to peptide separation, protein recovery is a great concern in the solution platform. Hydrophobic proteins are more difficult to recover from RPLC, mainly due to higher affinity of the hydrophobic proteins for the stationary phase than for the solvent in the mobile phase; [70] moreover, [71] IEC has questionable recovery of certain proteins. [71] Strategies for size separation in solutions of proteins, such as centrifugation and size exclusion chromatography, provide poor resolution. [16] In order to overcome these limitations, an effective solution-based separation known as GELFrEE was developed by Tran and Doucette [34] which separates intact proteins in solution according to their molecular weight, while maintaining high protein recovery. The ability of GELFrEE to maintain the protein in solution is provided in part by its use of SDS. In this prefractionation system, proteins can be subjected to top-down or bottom-up experiments. Unfortunately, the low concentration of SDS that is present in GELFrEE (~0.1%) is a concern with follow-up proteomics techniques such as MS, LC and LC/MS analysis. Thus, if GELFrEE is to be an effective separation platform in the proteomics experiment, strategies for SDS removal or alternative MS compatible ionic surfactants must be provided. Such a strategy would enhance the detection workflows of both bottom-up and top-down proteomics.

1.8 Research overview

This thesis presents efforts towards the development and evaluation of alternatives to SDS as well as techniques for removal of SDS for analysis of intact membrane proteins

using mass spectrometry-based detection. Here, the aim is to characterize MS-compatible additives for membrane protein analysis. These hydrophobic proteins, as described earlier, are widely studied due to their importance for biomarker and drug discovery. The methods evaluated and presented in this thesis cover alternatives to solubilize and/or resolubilize intact membrane proteins as well as distinct aspects of the current SDS removal protocols prior to LC/MS-based analysis.

Considering the limitation of SDS in a proteome analysis workflow, numerous SDS substitutes that are MS compatible have been proposed. For example, some MS-compatible detergents like RapiGest and PPS (sodium 3-(4-(1,1-bis(hexyloxy)ethyl)pyridinium-1-yl)propane-1-sulfonate) have been exploited for membrane protein solubilization. [72] However, some hydrophobic peptides might be co-precipitated with those hydrolyzed by-products of these reagents. [73] Other additives such as alcohol (*e.g.* 60% methanol [74] and 50% trifluoroethanol [75]) are also alternatives to SDS for dissolving membrane proteins, but solvents that are highly concentrated in alcohol often inhibit the activity of enzymes in the subsequent digestion process. Among these MS-compatible additives, fluorinated surfactants and in particular PFOA (perfluorooctanoic acid) and its ammonium salt APFO (ammonium perfluorooctanoate) have been reported for solubilizing membrane proteins. [39] Considering MS tolerance, Ishihama *et al.* [38] classified PFOA as ESI-MS compatible, and therefore they designated this fluorinated surfactant as a potential substitute for SDS since this compound has strong surfactant properties.

Chapter 2 explores the effects of both PFOA and APFO on proteome experiments, comparing its utility to that of the more commonly used detergent SDS. The compatibility of fluorinated surfactants is established for both reversed-phase liquid chromatography, as

well as ESI mass spectrometry, and for the combined LC/MS experiment. While it is assumed that the ESI source is sufficient to eliminate the surfactant (*i.e.* evaporation at high temperature), my results demonstrate that the presence of other ionic species (*e.g.* Na^+) can interfere with the removal process. In other words, APFO is not particularly tolerable to MS. Regardless, APFO will interfere with chromatographic separation and must be removed from the sample prior to analysis. We therefore perform an in depth analysis of the removal efficiency of APFO during solvent evaporation at reduced pressure (*i.e.* in a SpeedVac).

The removal of SDS is critical for the successful analysis of the peptides and/or proteins. Conventional methods such as dialysis, ion-exchange, gel filtration and protein precipitation with organic solvents are utilized for SDS removal. [35-37] However, all of these methods have the similar disadvantage that they may result in a significant loss of sample during protein sample cleanup. Puchades *et al.* [76] compared common procedures to remove SDS, including protein precipitation with acetone and chloroform/methanol/water (CMW). Although both CMW and acetone methods sufficiently reduce SDS prior to mass spectrometry, protein recovery of only 50% and 80% were obtained with CMW and acetone, respectively. [76] In other words, though protein purification appears possible with these precipitation strategies, this purity appears to come at the expense of a high degree of protein sample loss. It would be desirable to maintain minimal loss of protein sample during removal of SDS.

In Chapter 3, the efficiency of protein recovery for both CMW and acetone methods is performed in the context of a comprehensive proteome analysis, including the recovery of membrane proteins. Following precipitation of proteins, strategies to re-solubilize the

intact membrane proteins are also explored from the point of view of quantifying protein loss. Through this evaluation, it is demonstrated that extremely high protein recovery is possible following protein precipitation, contrary to the current understanding of CMW and acetone precipitation. By establishing high recovery with these precipitation methods, a novel strategy for intact membrane protein analysis through MS detection is also developed and validated.

Chapter 2

Perfluorooctanoic acid and ammonium perfluorooctanoate: Volatile surfactants for proteome analysis? [1]

2.1 Introduction

The ionic surfactant sodium dodecyl sulfate (SDS) is commonly employed to improve protein solubility or impart electrophoretic separation of a proteome (*e.g.* SDS PAGE). [77] Unfortunately, ionic surfactants generally suppress ESI-MS signals of proteins and peptides, [31] and are detrimental to RPLC separation of these compounds. [78] Notwithstanding potential improvements in MALDI-MS at high SDS concentration, [30] the threshold tolerance of SDS in the conventional (bottom-up) LC/ESI-MS experiment is far below its CMC (~0.24%). [29, 79]

To overcome the limitations of SDS in a proteome analysis workflow, one may attempt to eliminate the surfactant from the sample prior to LC/MS. [80] Despite an abundance of options for SDS removal, [29, 35-37, 76, 80] elimination of the detergent is still considered problematic. Thus, with focus on MS compatibility, numerous SDS substitutes have been proposed. [38, 81, 82] Among these is the class of fluorinated surfactants. Fluorinated surfactants are both hydrophobic and lipophobic, and are extremely effective at lowering water surface tension. Perfluorooctanoic acid (PFOA) was used industrially, until health concerns forced a phasing out of this environmentally persistent chemical. [40] The attraction of PFOA to the proteomics experiments relates to its high volatility (5.2 Pa

[1] A version of this chapter has been published in *Rapid Commun. Mass Spectrom.* (2012), 26, 523-531. Andrew Crowell contributed to the generation of Figure 2.2 by preparing the standards for analysis.

at 27 °C, rising to 40.7 Pa at 45.7 °C). [83] Consequentially, fluorinated surfactants have been dubbed MS-compatible. [38]

Shepherd and Holzenburg first introduced ammonium perfluorooctanoate (APFO) to proteome processing as a surfactant for solubilizing membrane proteins. [39] APFO was found extremely effective for a wide range of membrane systems at or above 2% APFO (~2-times its CMC). [39] Ramjeesingh *et al.* later substituted SDS for PFOA (as the sodium salt) in a gel electrophoresis separation of membrane proteins (dubbed PFO/PAGE). [84] Most recently, Kadiyala *et al.* reported a 'single-tube' shotgun proteomics method using PFOA. [18] The authors demonstrated the efficiency of the surfactant (in an ammonium bicarbonate buffer) for solubilization of membrane proteins. The detergent was also shown to be compatible with tryptic digestion (up to 0.5%), and could be removed from the sample prior to bottom-up MS analysis through a multistep evaporation strategy. [18]

Considering MS tolerance, Ishihama *et al.* classified PFOA as ESI-MS compatible, [38] attributing the counter-intuitive minimal signal suppression of this ionic surfactant [31] to the high volatility of PFOA. [38] Indeed, a direct MS infusion of the peptide, angiotensin II, in 30 mM PFOA (~1.2% w/v), caused essentially no drop in signal; the protein myoglobin retained 21% signal intensity in 25 mM surfactant. [38] PFOA has also been used for MALDI-MS analysis of membrane proteins, showing an enhancement in ionization when included at concentrations of 1%. [41]

The ammonium salt, APFO, has also been dubbed a volatile (and thus MS-compatible) surfactant. [85] In two independent studies by Bottaro and by Pettersson, APFO was shown to be a compatible substitute for SDS during micellar electrokinetic chromatography (MEKC)/ ESI-MS. [85, 86] It is worth noting that the volatility of APFO is

approximately 3 orders of magnitude lower than the acid form, PFOA. [87] The ionization source in MEKC/ESI-MS differs considerably from the conventional LC/ESI source in that a large makeup flow (*e.g.* methanol) acts to dilute the sample prior to ESI. Considering this makeup flow, a study by Somsen *et al.* concluded that SDS causes minimal MS signal deterioration in MEKC/ ESI-MS. [88] The level to which APFO is compatible with protein/ peptide analysis *via* ESI-MS has not been established.

Fluorinated surfactants will also influence the chromatographic separation of proteins and peptides, another critical aspect of the proteomics experiment. Fluorinated surfactants are popular substitutes for TFA as ion-pairing agents in RPLC. [89, 90] PFOA has been used to modify the separation behavior of small, hydrophilic molecules (*e.g.* amino acids), and of some peptides, particularly when such compounds exhibit poor RPLC retention. Petritis used PFOA at a concentration of 0.5 mM to improve separation of amino acids. [91] Ishihama concluded that PFOA outperforms TFA as an ion-pairing reagent for peptides. [38] By contrast, Kadiyala observed a broadening of chromatographic peaks when residual APFO (~0.012%) was present in the sample. [18]

Given the interest in 'volatile' (MS-compatible) fluorosurfactants, a detailed investigation of the tolerance threshold of LC and LC/MS experiments towards APFO is warranted. Here, the APFO tolerance was found to be superior to SDS, but this increased MS tolerance is insignificant when considering the higher working concentration of APFO required for equivalent solubilization efficiency. Nonetheless, APFO can be removed prior to LC/MS through a single (one-step) evaporation from acidified solvent.

2.2 Experimental

2.2.1 Materials

Myoglobin (Cat. # 100684-32-0) as well as TPCK-treated trypsin (Cat. # T8802) were purchased from Sigma Aldrich (Oakville, Canada). Substance P (Cat. # 24279) and bradykinin (Cat. # 20667) were acquired from AnaSpec, Inc. (Fremont, USA). Perfluorooctanoic acid (PFOA, Cat. # 171468) and ammonium perfluorooctanoate (APFO, Cat. # 77262) were from Sigma Aldrich, while SDS (Cat. # 161–0302) was from BioRad (Mississauga, Canada). Solvents of HPLC grade were obtained from Fisher Scientific (Ottawa, Canada), with Milli-Q water purified to 18.2 MΩ cm. All other reagents were from Sigma Aldrich and were used without further purification.

2.2.2 Yeast proteome extraction

S. cerevisiae (BY4741), gratefully donated by Dr. Melanie Dobson (Dalhousie University, Halifax, Canada), was grown in YEPD (Yeast Extract Peptone Dextrose) media at 25 °C and harvested at an optical density (OD) of 0.5 by centrifugation at 3500 × g for 5 min. The pellet was washed in PBS buffer and collected by centrifugation at 3200 × g for 15 min at 4 °C. The yeast pellet was transferred to liquid nitrogen, and the frozen cells were ground for 10 min under liquid nitrogen using a mortar and pestle. Proteins were extracted with 3 mL of 50 mM Tris (pH 8) plus protease inhibitors from Sigma (Cat # 2714) with gentle shaking for 20 min at room temperature, followed by a 5 min centrifugation at 13000 rpm on a benchtop centrifuge (Fisher Scientific, Ottawa, Canada) to clarify the extract. The final protein concentration was 3.9 g/L, as determined using a bicinchoninic acid (BCA) total protein assay (Pierce, Rockford, IL, USA).

2.2.3 Protein resolubilization

Extracted yeast proteins were precipitated overnight at -20 °C following addition of acetone in a 4:1 ratio over the sample as described previously. [29] The resulting protein

pellet was washed once with 400 μL of cold acetone. The pellet was resuspended in 100 μL water with increasing concentrations of APFO, SDS or PFOA, as described in Section 2.3.1 (triplicates per surfactant concentration). All samples were then sonicated for 30 min and allowed to sit on the benchtop overnight at room temperature. Samples were then centrifuged for 15 min at 13000 rpm using the benchtop centrifuge to sediment any undissolved protein and a BCA assay was conducted on both the top (50 μL) and bottom (50 μL) half of each sample.

2.2.4 APFO removal by evaporation

Evaporation experiments were conducted with a Savant SpeedVac concentrator, connected to an Edwards RV8 rotary pump through a liquid nitrogen cold trap. Following initial pump down (a few minutes) the pressure reading during evaporation stabilized at a constant 100 mTorr. Samples were evaporated from 1.5 mL polyethylene vials (Fisher Cat. No. 05-408-129), starting from an initial volume of 100 μL aqueous buffer per vial (see Results and Discussion section for buffer composition), with triplicate samples per time point, as defined in Section 2.3.5.

2.2.5 APFO quantification

A calibration curve was constructed using the selected negative ion peak areas for APFO (*m/z* 412.8) as monitored on an LCQ Duo ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) following elution from a RPLC column. APFO-containing samples were prepared in 67% acetonitrile with 0.1% formic acid/water, and analyzed by injection of 5 μL (in triplicate) onto a self-packed 50 × 1 mm i.d. C_{12} column (4 mm Jupiter beads, 300 Å pore size; Phenomenex, Torrance, CA, USA). The HPLC solvent system (67% acetonitrile, 0.1% formic acid, water) was pumped at a flow rate of

15 μL/min, with splitless coupling to ESI-MS. The APFO ion was monitored using the following MS operating parameters: Spray voltage: 4 kV; capillary temperature: 250 °C; capillary voltage: –38 V; tube lens offset: 25 V; number of microscans: 3; maximum inject time: 200 ms; scan range: *m/z* 360 to 420. Test samples were diluted prior to LC/MS analysis such that signals were within the linear range of the calibration curve (1.16×10^{-4} to 1.16×10^{-2} mM APFO).

2.2.6 Direct infusion ESI-MS analysis

Peptide and protein standards were prepared at the specified concentration in a solvent system comprising 50% methanol, along with the appropriate concentration of APFO or of SDS (see Results and Discussion section). Unless otherwise specified, the solvent system included 0.1% formic acid. Either 5 or 10 μL were injected into the LCQ Duo ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) at a rate of 20 μL/min, with 50:50 methanol/water as carrier solvent. Unless otherwise specified, MS operating parameters were as described above, with exception to the scan range (*m/z* 50 to 2000). The reported signal intensities represent the average peak areas from triplicate injections.

2.2.7 LC/UV analysis

Aqueous samples of substance P (100 μL, 0.2 g/L), containing the appropriate concentration of APFO or SDS, were subjected to LC separation on a 10 mm × 0.5 mm self-packed C_{18} column (5 mm beads; Waters Corporation, Milford, MA, USA). An Agilent 1200 HPLC system was used with UV detection at 214 nm. The flow rate was 100 μL/min. Solvent A was water plus 0.1% trifluoroacetic acid (TFA) and solvent B was acetonitrile with 0.1% TFA. The gradient was initially set to 5% B, and adjusted as follows:

40% B by 20 min, 95% by 30 min, 5% by 40 min, 95% by 50 min; drop to 5% at 60 min. The total run time was 70 min.

2.2.8 Trypsin digestion and sample clean up

Yeast proteins (1 g/L) were subjected to overnight tryptic digestion (50:1 mass ratio of protein: trypsin), following reduction and alkylation with dithiothreitol (DTT) and iodoacetamide as described previously. [29] The resulting digest was acidified with 10% TFA and evaporated in a SpeedVac, then reconstituted in 0.1% aqueous TFA. The reconstituted peptides were subjected to automated sample cleanup, as described previously. [29] A 1 × 50 mm column with 5 μm C_{18} beads (Waters Corporation, Milford, MA, USA) was used on an Agilent 1200 HPLC system. Peptides were collected as a single fraction, which was then evaporated in a SpeedVac, and frozen at -20 °C until just prior to LC/MS/MS analysis. Samples were re-suspended in 0.1% formic acid/water, along with the appropriate concentration of APFO (0 to 4.6 mM) for LC/MS/MS analysis.

2.2.9 LC-MS/MS, bottom-up analysis and data searching

Digested yeast proteins (10 μL, 1 μg per injection) were subjected to LC/MS/MS analysis, with triplicate injection per sample. The column was a self-packed 20 cm × 75 μm spray tip (New Objective, Woburn, MA, USA) using 4 μm C12 Jupiter beads (Phenomenex, Torrance, CA, USA). The flow rate was 0.3 μL/min. Solvent A was water with 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. The gradient was initially set to 5% B, and adjusted as follows: 7.5% by 0.1 min; 20% by 90 min; 25% by 115 min; 35% by 120 min; 80% by 121 min; drop to 5% at 125 min.

Peptide identification was on an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) employing a ‘triple play’ data-dependent scan. 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. Charge state screening was enabled to ignore singly charged ions, ions with a charge of 4 and greater, or ions where the charge state could not be assigned. MS operating parameters were as follows: Mass range: *m/z* 400 to 1300; activation type: CID; normalized collision energy: 35; scan events: 2 and 3 repeated for top 3 peaks.

Database searching was executed according to the MS/MS spectra searched against the *Saccharomyces cerevisiae* yeast proteome using Bioworks browser software package from Thermo Fisher, which uses the SEQUEST search engine. Data was filtered as follows: $\Delta Cn \leq 0.1$; RSP ≥ 4; Number of top matches equals 1; Peptide probability $\leq 5 \times 10^{-3}$; Unique peptides. The +2 ions required an X_{corr} of at least 2.2 and +3 ions at least 3.75. These criteria establish a peptide false positive rate of 1%, through reverse database searching.

2.3 Results and Discussion

2.3.1 Protein solubilization with surfactants

Surfactants are often employed to facilitate protein extraction and/or solubilization. Fluorinated surfactants have been introduced as a suitable class of biological surfactants, with reference to PFOA for proteome solubilization. To clarify, previous work has described the utility of sodium or ammonium *salts* of PFOA for membrane protein solubilization. [18, 39, 84] The *acid* form of PFOA has yet to be demonstrated for proteome solubilization.

The acetone precipitate of yeast whole cell protein extract was selected as a test sample, given its resistance to solubilize in pure aqueous solvent (Figure 2.1). As one

predicts, protein recovery increases with inclusion of more SDS in the solvent. Complete protein solubilization was achieved at or above 3.5 mM SDS; recall the CMC of SDS in pure water as 8.2 mM. [92] By comparison, complete protein solubilization is obtained at or above an APFO concentration of 11.6 mM (CMC = 13 to 27.5 mM, depending on solvent composition). [39] Thus, at a concentration approximately three times higher than that used with SDS, the solubilization potential of APFO is equivalent to that of SDS. Previous work on APFO for membrane protein solubilization recommended a concentration of 4% w/v, or 92 mM APFO. [39] Though higher than reported here, the concentration differences may be attributed to the nature of the samples being solubilized.

While APFO is an effective protein-solubilizing additive, PFOA is ineffective at facilitating protein resolubilization from an acetone precipitate (Figure 2.1). Relative to pure water, the percentage of resolubilized protein decreased above approximately 10 mM PFOA. This decrease may be a consequence of the reduced pH of the solvent upon addition of the weak acid. The highest concentration of PFOA tested from Figure 2.1 (25 mM) corresponds to its solubility limit in water. Thus, while PFOA and APFO are both classified as surfactants, only the salt form is shown effective at assisting protein solubilization following acetone precipitation.

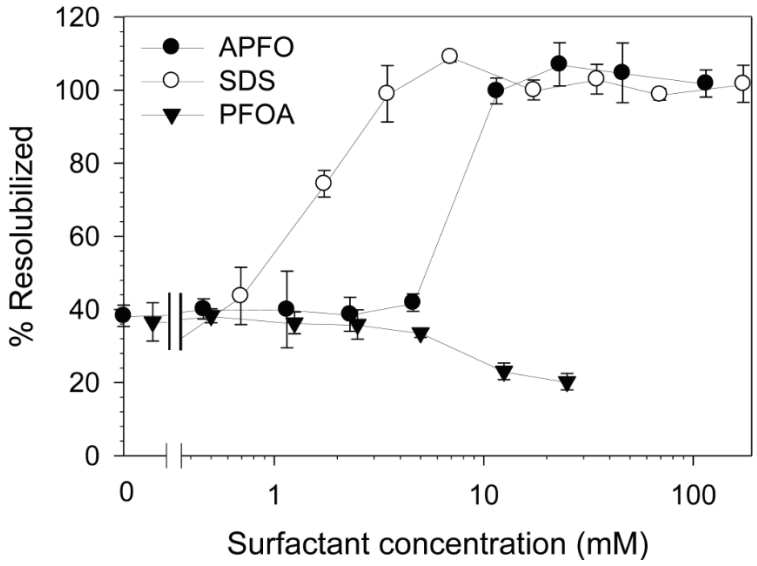

Figure 2.1 The solubilization potentials of APFO, SDS and PFOA are demonstrated with an acetone-precipitated yeast (whole cell) proteome extract. The percent resolubilized was determined by BCA assay of the top and bottom half of a centrifuged sample following sonication and overnight incubation in the solvent. Error bars represent the standard deviation of the mean associated with triplicate analysis per condition. Lines connecting points on the graph are added purely to facilitate visualization. Surfactant concentrations are expressed in molarities to account for differences in molecular weight of the surfactants.

2.3.2 Influence of APFO in MS infusion analysis

Previous work has labeled PFOA as an MS-compatible surfactant. [38] However, the compound does not directly facilitate protein solubilization. Given the lower volatility of APFO, [87] together with the presence of an ammonium cation, one might suspect the salt form to follow the behavior of other ionic surfactants, causing significant suppression of proteins and peptides in ESI-MS. A test sample consisting of a standard protein (myoglobin) and peptide (substance P) was selected to quantify the suppression of APFO in a direct infusion ESI-MS study. MS suppression is influenced by other variables, including solvent composition, sample concentration, flow rate, as well as the nature of the ESI source. Thus, the suppression effects of APFO were quantified relative to that of the more common SDS, with results summarized in Figure 2.2. To facilitate comparison, a

'tolerance threshold' is defined, being the surfactant concentration which retains MS signal intensity above 50% relative to the control sample (in the absence of surfactant).

From Figures 2.2(A) and 2.2(B), the MS tolerance threshold of APFO occurs at a higher concentration than SDS. Stated differently, under identical conditions, direct infusion ESI-MS analysis tolerates more APFO than SDS before 50% suppression is observed. However, this higher APFO concentration is viewed as minimally significant, considering that the working concentration of SDS is lower than that of APFO (Figure 2.1). A tolerance threshold is observed at an SDS concentration of 0.69 mM for substance P, and 0.17 mM for myoglobin. A lower tolerance to surfactants is to be expected for proteins as they are generally more susceptible to suppression than are peptides. By comparison, APFO can be added to samples at approximately twice the concentration of SDS (1.16 mM for substance P, or 0.46 mM for myoglobin), before 50% suppression is observed. Figure 2.2(C) illustrates the quality of the MS spectrum observed in 1.16 mM APFO. The myoglobin signal intensity represents 15% of the control sample. Though a charge state envelope is still observable, there is a noted drop in intensity. While this observation may suggest that APFO is more amenable to the electrospray process, from a practical standpoint, APFO must be added at approximately three times the concentration of SDS to achieve a similar degree of solubilization (Figure 2.1). Excluding sample dilution, this 'working' concentration is well above the tolerance threshold of ESI-MS. Furthermore, one observes from Figure 2.2(B) that suppression of APFO overtakes that of SDS at high concentrations (beyond 2.3 mM APFO).

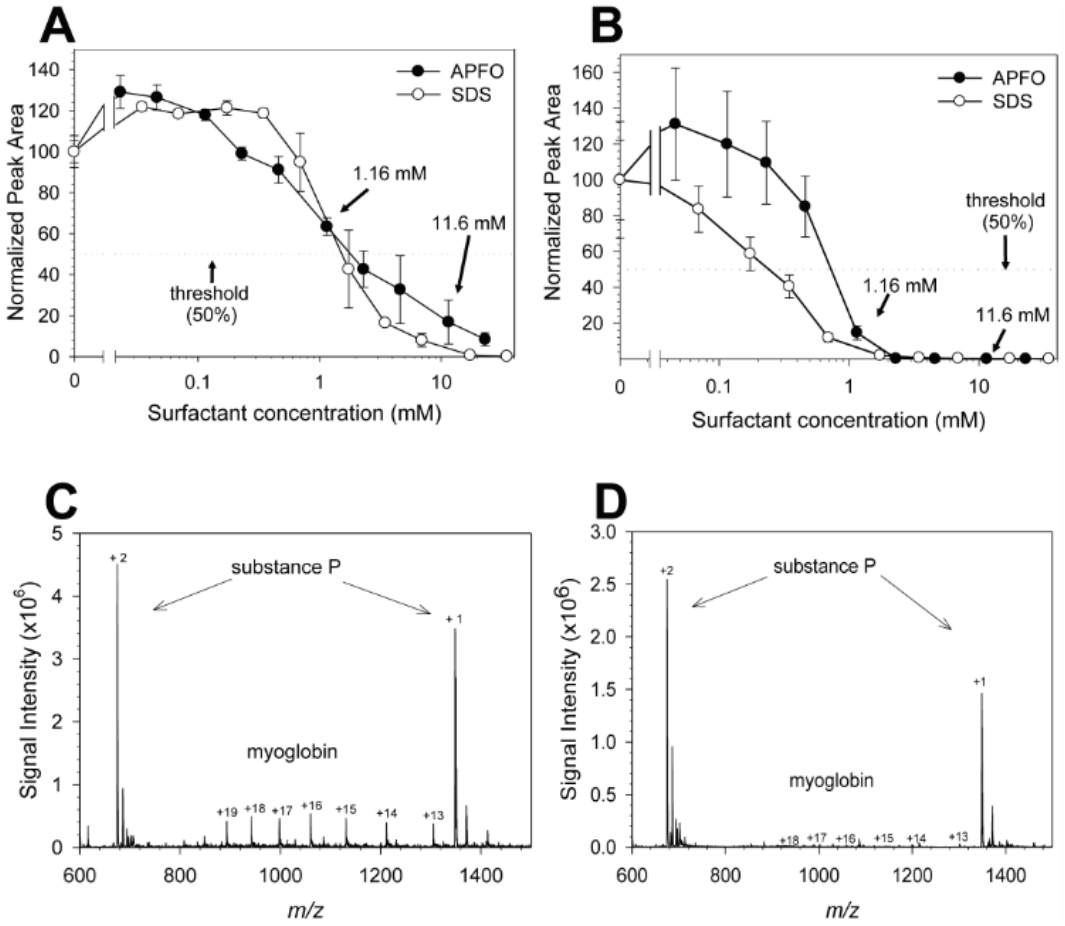

Figure 2.2 Suppression effects of APFO and SDS observed through flow injection ESI-MS for a mixture of (a) substance P and (b) myoglobin. Sample conditions: protein and peptide concentration at 0.5 mg/mL; solvent 50% methanol, 49.9% water, 0.1% formic acid along with specified concentration of surfactant; flow rate 20 µL/min; injection volume 5 µL (MS conditions provided in experimental). Samples were run in triplicate (error bars = standard deviation of means), with peak areas determined from summed extracted traces of the most prominent ions: substance P (*m/z* 674.9 ± 0.5, 1348.7 ± 0.5); myoglobin (*m/z*: 998.1, 1131.0, 1211.8, 1304.3 ± 0.5), respectively. The 50% suppression threshold is indicated in (a) and (b). Representative mass spectra, summed over the injection of a sample containing (c) 1.16 mM APFO and (d) 11.6 mM APFO, illustrate the extent of MS suppression.

At 0.5% APFO in the sample (*i.e.* 11.6 mM), the spectral quality of myoglobin is extremely poor, as shown in Figure 2.2 (D). This figure serves to illustrate that APFO will cause almost complete signal suppression at high concentration. Thus, based on these results, APFO should not be classified as a 'MS-compatible surfactant' for proteome

processing. It is noted that MS signals are fully restored following infusion of the APFO surfactant at high concentration. Similar to other non-volatile components, repeated use of the surfactant warrants cleaning of the instrumental source.

2.3.3 APFO *vs* PFOA in MS infusion analysis

The potential for significant MS suppression at high APFO concentration contrasts with previous studies which reported the compatibility of fluorinated surfactants with MS. [38, 85, 86] Minimal signals were observed for peptides and proteins, including angiotensin II (results not shown), and reinforce a conclusion that APFO is not amenable to direct MS infusion at high concentration. The poor tolerance of APFO may be attributed to the lower volatility of the salt, or to the presence of the ammonium counterion in the sample. It should be clearly stated that the APFO suppression study described here was conducted in acidified solvent (0.1% formic acid). At the highest concentration of APFO plotted in Figure 2.1, 23.2 mM, formic acid would be present at an approximate 1:1 mole ratio relative to APFO. The pKa for PFOA has been debated. [93-95] Given that species concentrate during electrospray to different extents, together with the volatile nature of the compounds involved (ammonium formate, perfluorooctanoic acid), it would be difficult to calculate the ratio of protonated/deprotonated PFOA within the ESI process. Nonetheless, it can be speculated that the sample pH may influence the suppression effects caused by this surfactant.

The signal intensity of a peptide (bradykinin) was monitored in a direct infusion ESI-MS experiment, as a function of the acid and surfactant type in the sample. These results are summarized in Figure 2.3(A). As expected, for the control sample (no surfactant) with formic acid at 0.1% or 1%, no significant change in intensity of bradykinin was

observed. The inclusion of a strong acid, HCl (60 mM), caused significant suppression of the peptide signal ($p < 0.05$). [96] At 11.6 mM APFO, increasing the formic acid concentration to 1% (ca. 20:1 mole ratio over APFO) did not reverse the suppression caused by the surfactant. Inclusion of the strong acid (HCl), at an approximate 5:1 mole ratio over the surfactant, also did not improve MS tolerance towards APFO. Similarly, an approximate 1:1 ratio of HCl to APFO caused no significant change in suppression (results not shown). Equivalent results were observed with the protein myoglobin, demonstrating that the restoration of a peptide or protein signal in high concentrations of APFO is not observed by altering the acid content of the sample. Further attempts were made to improve MS signals, and included altering the conditions of the MS source. Specifically, the source capillary temperature was raised, and the nitrogen gas flow rate was increased, with intent on improving the removal of APFO (or PFOA) through evaporation. While a relative improvement in MS tolerance towards APFO could be obtained at higher source capillary temperature, such ESI conditions were non-optimal, and thus there resulted a net decrease in signal intensity compared to the control sample observed under optimal MS conditions (see Figure 2.4).

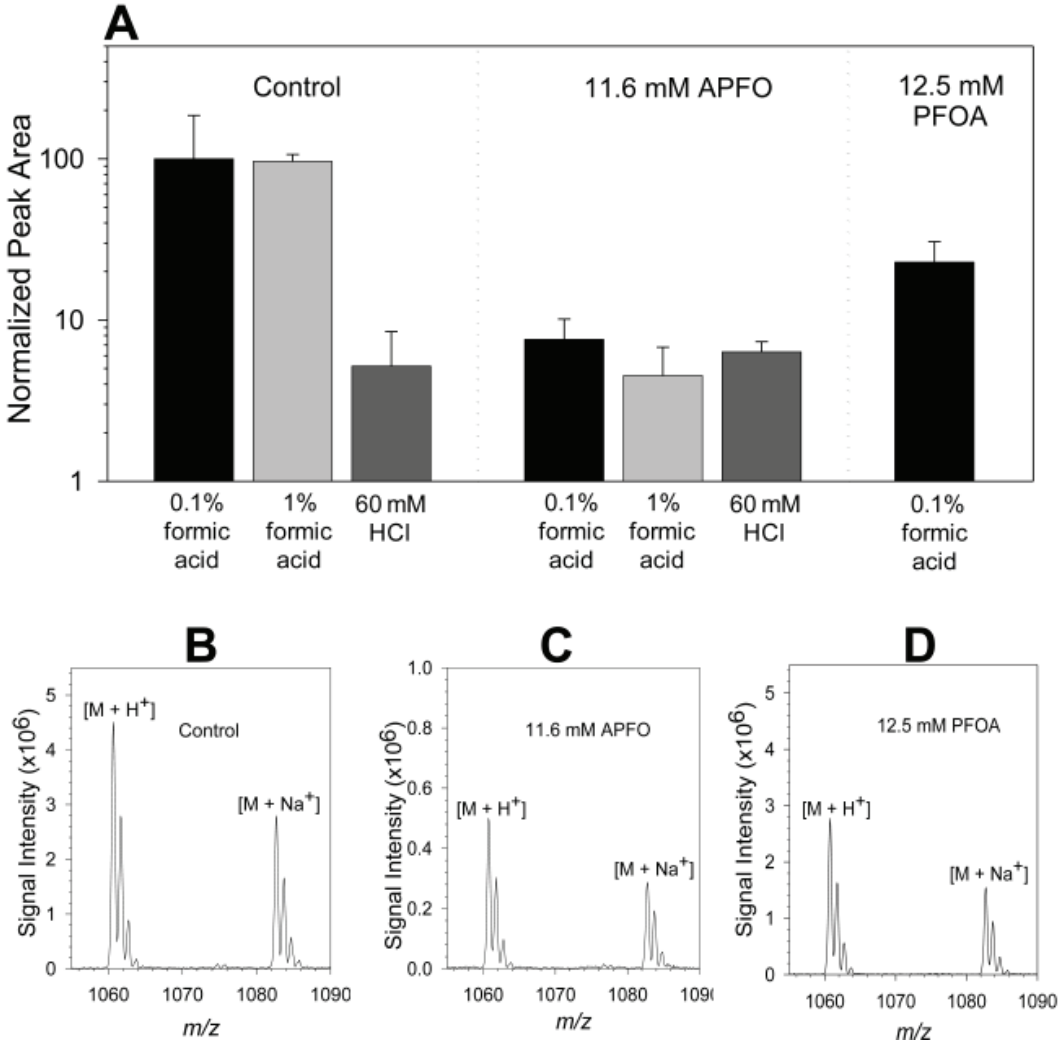

Figure 2.3 The normalized intensities of the extracted peak areas for bradykinin (sum of *m/z* 531, 1060.7 ± 0.5) are shown in (a), as a function of the surfactant and acid content in the sample (as indicated, control = no surfactant). Sample conditions: 0.1 mg/mL; solvent 50% methanol, water + specified concentration of acid and surfactant; flow rate 20 µL/min, injection volume 10 µL (MS conditions provided in experimental). Error bars represent the standard deviation of means from triplicate injections. Representative mass spectra, summed over the injection of a sample containing (b) no surfactant, (c) 11.6 mM APFO, or (d) 12.5 mM PFOA, illustrate the presence of the sodium adduct of bradykinin.

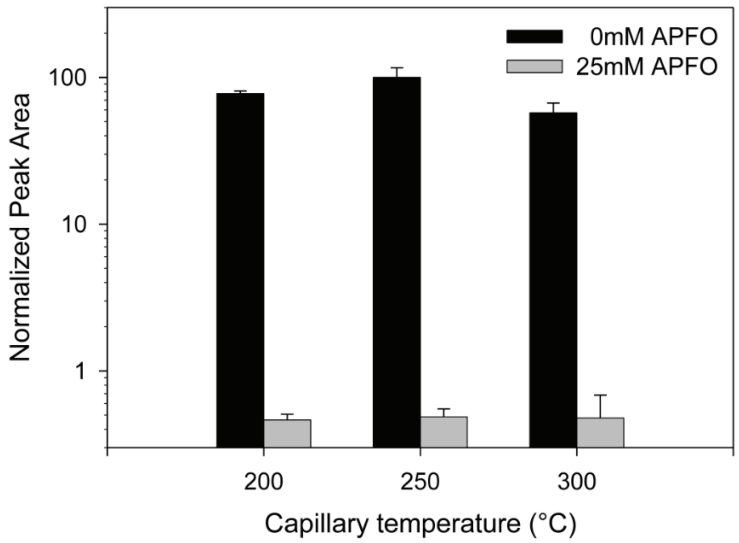

Figure 2.4 The normalized intensities of the extracted peak area for myoglobin (*m/z*: 998.1, 1131.0, 1211.8, 1304.3 ± 0.5) as function of the capillary temperature and APFO concentration. Sample conditions: 0.5 µg/µL; solvent 50% methanol, 49.9% water and 0.1% formic acid; flow rate 20 µL/min; injection volume 5 µL. MS parameters as described in the Experimental section. Error bars represent the standard deviation of means from triplicate injections.

The suppression behavior of the acid form, PFOA, was measured directly, using identical conditions described for APFO, with results summarized in Figure 2.3(A). Given its classification as a volatile surfactant, the results were somewhat surprising in that 12.5 mM PFOA caused severe MS signal suppression (see Figure 2.5) for the peptide bradykinin (also confirmed with angiotensin II). Minor differences in suppression behavior are to be expected, given variations in instrumental platforms. However, it is unlikely that such variations account for the contrasting suppression of PFOA reported here relative to previous studies. [38, 85, 86] Thus, an alternative explanation for suppression behavior was sought, and may be provided through the MS spectra shown in Figures 2.3(B)–2.3(D). Here, the presence of sodium adducts are clearly observed, indicating that sodium is present in the system (*i.e.* from the sample, or as a residual in the source). It is interesting to note that the ammonium adduct was not observed (Figure 2.3(C)), despite addition of the

ammonium salt to the sample. Nonetheless, the presence of sodium suggests that sodium perfluorooctanoate may have formed, which would have minimal volatility. In previous studies, sodium adducts have been noted to dominate the spectrum of SDS-containing samples, contributing to the overall decrease in signal intensity. [97] The ammonium salt of dodecyl sulfate has been demonstrated as a favorable substitute. [97] Further evidence of the deleterious effects of sodium is provided in Figure 2.6. A synergistic effect was observed whereby the suppression behavior of APFO + NaCl was greater than the suppression of the two additives alone.

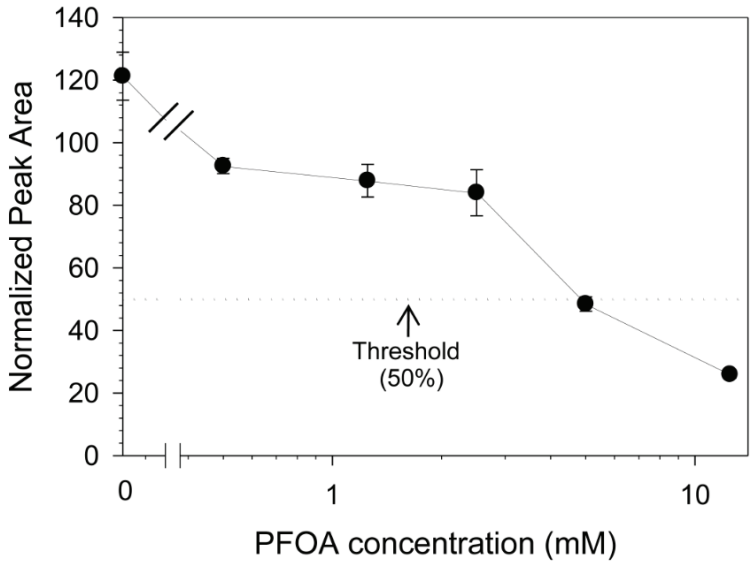

Figure 2.5 Suppression effects of PFOA observed through flow injection ESI-MS for bradykinin (*m/z* 531, 1060.7 ± 0.5). Sample conditions: 0.1 µg/µL; solvent 50% methanol, 49.9% water, 0.1% formic acid along with specified concentration of surfactant; flow rate 20 µL/min; injection volume 10 µL (MS parameters described in experimental). Samples were analyzed in triplicate (error bars = standard deviation of means), with peak areas determined from summed extracted traces of both bradykinin peaks cited above. The 50% suppression threshold is indicated.

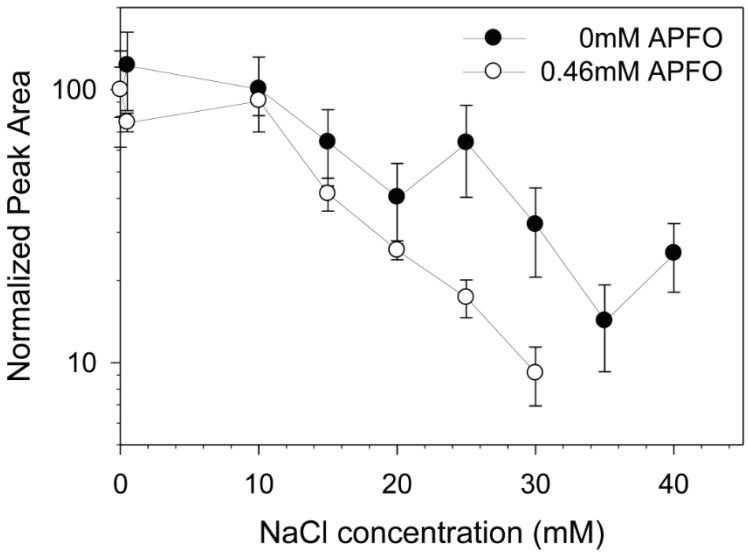

Figure 2.6 The normalized intensities of the extracted peak areas for myoglobin peaks (*m/z*: 998.1, 1131.0, 1211.8, 1304.3 ± 0.5) are shown as function of salt and surfactant concentration. Sample conditions: 0.5 µg/µL; solvent 50% methanol, 49.9% water, 0.1% formic acid along with specified concentration of NaCl; flow rate 20 µL/min; injection volume 5 µL (further details provided in the Experimental section). Samples were analyzed in triplicate (error bars = standard deviation of means).

2.3.4 APFO in the LC/MS experiment

Flow injection MS is an unlikely experiment in a proteome workflow; the coupling of RPLC to ESI-MS constitutes the typical proteomic experiment. Contrary to the classification of fluorosurfactants as 'MS-compatible', Kadiyala reported that even residual traces of PFOA (ca. 0.14 mM) in a peptide mixture caused significant deterioration of LC/MS; the deterioration was attributed to the broadening of chromatographic peaks. [18] The effects of SDS on RPLC separation are well known; [78, 98] Chen *et al.* demonstrated the retention of tryptic enolase to be modified in the presence of SDS, and also reported the increased peak width and decreased peak height of a protein upon addition of SDS to a chromatographic separation. [99]

Figures 2.7 to 2.9 provide a quantitative assessment of the reduced LC performance at high APFO concentrations. Above 0.46 mM APFO, the surfactant causes a significant shift of peptides towards higher retention times ($p = 0.0008$). This is observed in Figure 2.7, as the substance P retention continues to shift to later elution times. Co-elution of the surfactant was confirmed through negative mode ESI-MS (results not shown), and are indicative of a surfactant-peptide complex which would possess higher hydrophobic character. Similar to the flow injection MS experiment, Figure 2.7 demonstrates RPLC to tolerate a modestly greater concentration of APFO (~2-fold higher) relative to SDS, before the retention time shift is observed. It is again noted that a lower working concentration of SDS negates the improved tolerance of APFO during RPLC. Along with the shift in retention, there is a corresponding increase in peak width (Figure 2.8) and decrease in peak height as well as peak area (Figure 2.9). The decreased peak area, as determined by ESI-MS analysis, is in keeping with the suppression behavior of the surfactant. Thus, high concentrations of APFO in the sample have negative consequences on both chromatographic separation of peptides, and subsequent MS analysis of the eluting peaks.

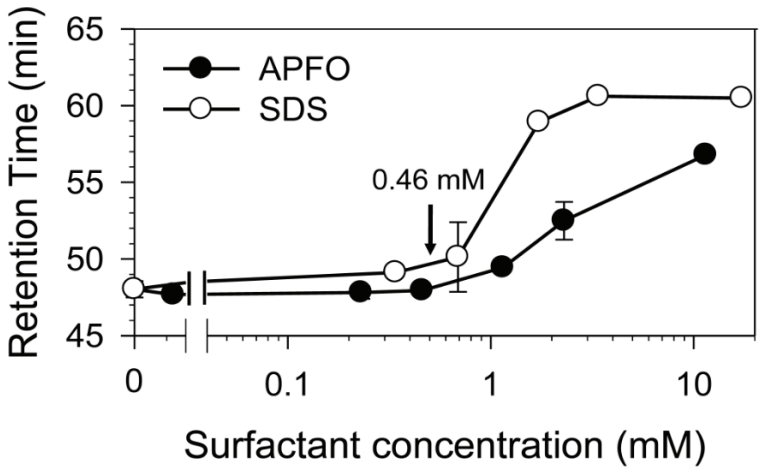

Figure 2.7 Demonstrates the shift in retention observed by LC/UV upon increase of the surfactants APFO or SDS to a sample of substance P. Sample conditions: 0.2 mg/mL, 100 µL injected, C_{18} column (10 cm × 0.5 mm) at flow rate of 100 µL/min (further details provided in experimental). Error bars represent standard deviation of means from triplicate injections.

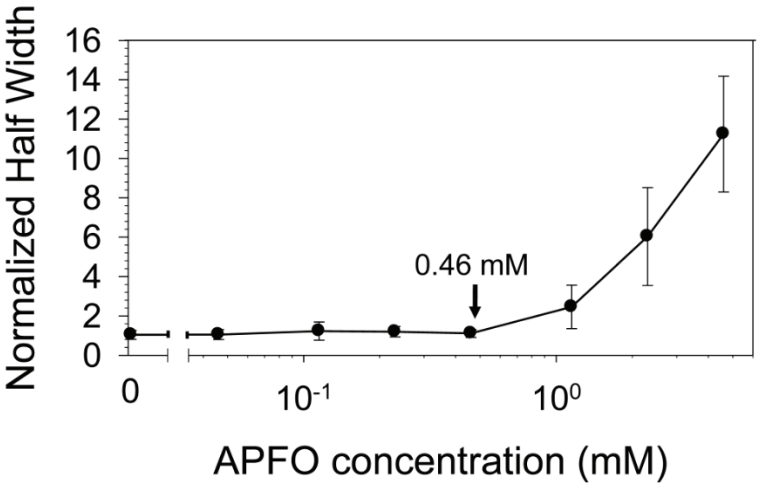

Figure 2.8 The influence of surfactants on the increase in peak width. Sample conditions: 0.1 mg/mL tryptic digest of yeast, 10 µL injection, 75 mm i.d. reversed-phase C_{12} column (20 cm × 75 mm) at 300 nL/min (further details provided in the Experimental section). Error bars represent standard deviation of normalized peak width from four representative yeast peptides (*m/z*: 910.4, 927.9, 508.7 and 493.7) observed over a single run under the conditions specified.

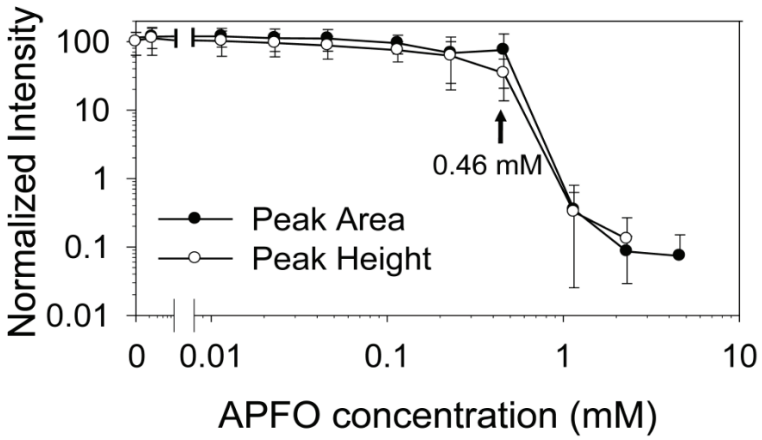

Figure 2.9 Illustration of the decreased peak height and peak width upon increase of APFO in the sample. Sample conditions: 0.1 mg/mL tryptic digest of yeast, 10 µL injection, 75 mm i.d. reversed-phase C_{12} column (20 cm × 75 mm) at 300 nL/min (further details provided in the Experimental section). Error bars represent standard deviation of normalized peak height/ area, from four representative yeast peptides (*m/z*: 910.4, 927.9, 508.7 and 493.7) observed over a single run under the conditions specified.

In a previous study, Botelho *et al.* reported on the upper threshold tolerance of SDS in a bottom-up experiment, which incorporates LC/MS/MS. [29] It was determined that 0.01% SDS (0.34 mM) could be tolerated without compromising the efficiency of protein identification. Under similar conditions, the tolerance threshold of APFO in a bottom-up MS experiment was determined through analysis of a tryptic yeast digest at increasing concentrations of APFO. As shown in Figure 2.10, a tolerance threshold of 0.46 mM APFO (0.02%) is again established, permitting reliable proteome analysis up to this level of surfactant. This level again corresponds to the concentration at which significant shift in retention time, peak width, and peak intensity is not observed (shown previously in Figure 2.7). Thus, for a proteome workflow, the established LC/MS/MS threshold provides a quantitative measure to which APFO must be reduced prior to proteome analysis. It is noted here that the APFO threshold is classified according to several variables, including for example the surfactant to protein ratio, the protein concentration and the volume of sample

injected. The threshold concentration is nonetheless significantly lower than the limit observed for effective proteome solubilization (*i.e.* 11.6 mM, as established in Figure 2.1). Thus, a method for APFO reduction (prior to LC/MS) becomes a critical aspect of the proteome workflow.

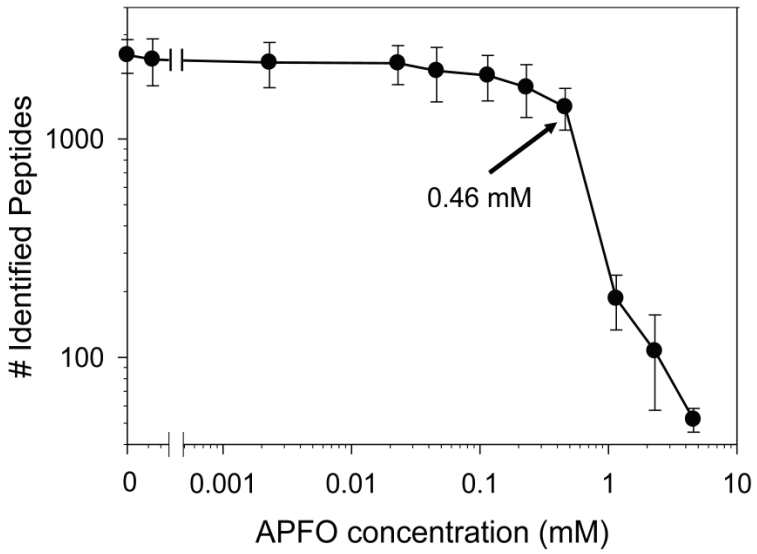

Figure 2.10 The number of yeast peptides identified from LC/ MS/MS injections of a tryptic digest (whole cell extract) containing increasing concentrations of APFO. Sample conditions: 0.1 mg/mL, 10 µL injected, 20 cm × 75 mm spray tip column at flow rate of 0.3 µL/min (further details provided in the Experimental section). Error bars represent standard deviation of means from triplicate injection of the sample at each specified condition.

2.3.5 Removal of APFO from a proteome sample by evaporation

It is common practice to remove surfactants prior to MS analysis. As a volatile surfactant, PFOA may be removed during a solvent evaporation step, being typical of a proteome experiment. Such a strategy has the benefits of minimizing protein loss, and has been previously demonstrated for PFOA. [18, 38] Though the volatility of APFO is significantly lower than that of PFOA, [87] the inclusion of excess (non-volatile) acid in the sample would favor formation of the protonated (more volatile) form of PFOA. Kadiyala

incorporated a small quantity of TFA to assist evaporation of PFOA from an ammonium bicarbonate buffer, [18] though it was found that six evaporation cycles were necessary to reduce the fluorosurfactant concentration to permit LC/MS/MS. This multi-stage drying strategy was repeated here, though it was found necessary to increase the cycle time from the recommended 1 h to a minimum 5 h per cycle, a consequence of higher pressure in the SpeedVac (ca. 100 mTorr in current study *vs* <10 mTorr as previously reported [18]).

An alternative, single-step evaporation strategy was devised. Knowledge of the threshold tolerance of APFO in a proteome experiment (<0.46 mM) provides a critical value to which the APFO must be eliminated. The quantity of APFO remaining in a test sample was assessed (negative mode ESI-MS) over a time-course study following evaporation in a SpeedVac. A concentration of 11.6 mM APFO (0.5%) was chosen in the initial sample, being the highest level of surfactant still tolerable to tryptic digestion. [18] Figure 2.11 summarizes the results, and reveals the improved efficiency of surfactant removal upon acidification of the sample. The addition of a strong acid, 50 mM HCl (final pH ~2), ensures formation of PFOA. With HCl, approximately 99% of the surfactant was removed following only 2 h evaporation. Reduction of the surfactant below LC/MS/MS tolerance was also confirmed without acidification of the sample (*i.e.* water, pH 7), though overnight evaporation (*i.e.* 16 h) was required. By contrast, in a buffer system comprising 50 mM Tris (pH 8), the level of APFO in the sample was not significantly reduced, despite 16 h of evaporation. This can be explained by the formation of non-volatile salts of the fluorosurfactant (APFO, or Tris-PFO). An equivalent trend to the Tris-containing sample was also observed with 50 mM NaCl in the sample (results not shown).

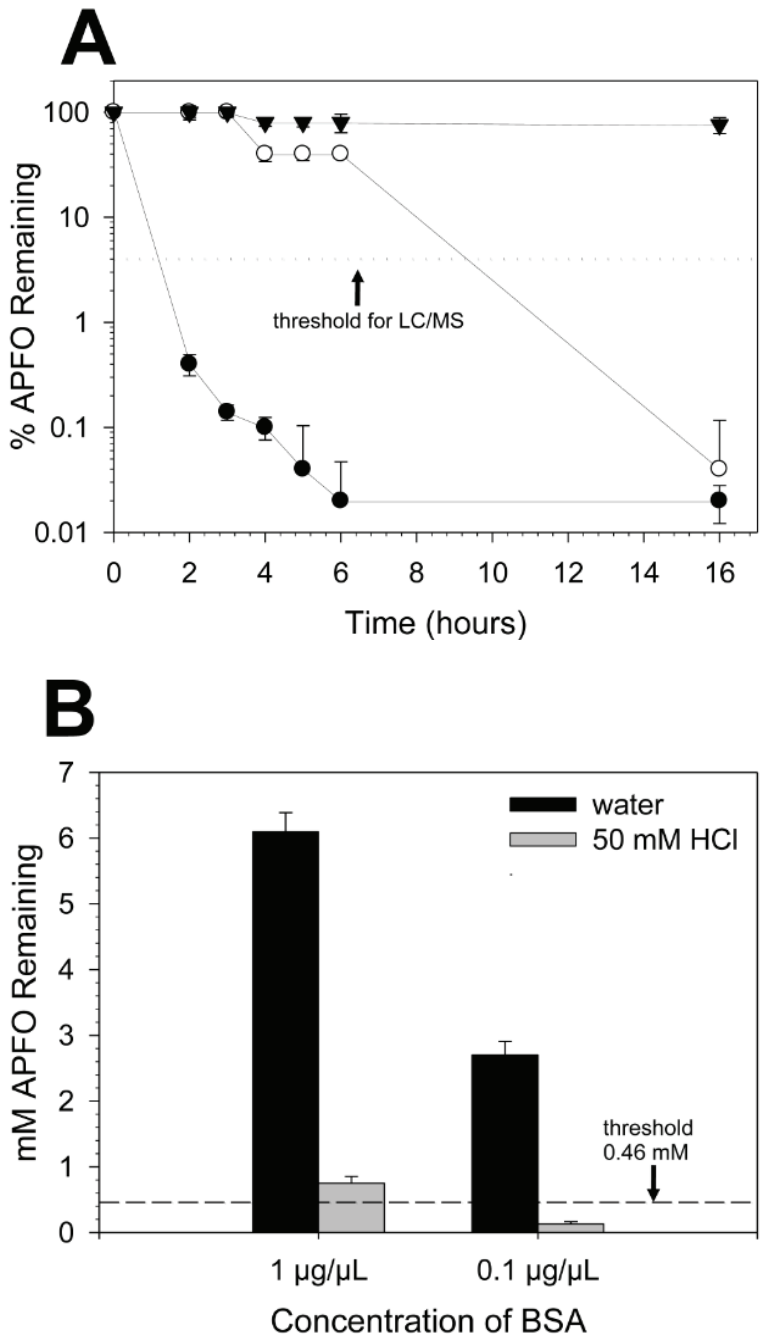

Figure 2.11 The level of APFO remaining in a sample (relative to initial concentration, *i.e.* 100%) is shown in (a), following evaporation in a SpeedVac (~100mTorr) for a specified period of time. Sample conditions: initial APFO concentration 11.6 mM; initial volume 100 µL; solvent composition (i) water (○), (ii) 50 mM HCl (●), or (iii) 50 mM Tris, pH 8 (▼). In (b), APFO evaporation includes the specified concentration of digested BSA, with 16 hours evaporation under acidic or neutral conditions. The level of APFO remaining is calculated following resolubilization to the initial sample volume.

As shown in Figure 2.11(B), the concentration of protein or of peptides in the sample affects the rate of evaporation of a fluorosurfactant; the affinity of the surfactant for these non-volatile components acts to diminish its volatility. An impure proteome sample may also contribute salts, which further decrease the volatility of the surfactant. The BSA digests added to the samples shown in Figure 2.11(B) constitute desalted samples (RPLC cleaned) and so the decreased volatility can be attributed to the affinity of the surfactant with the peptides. At BSA concentrations of 1 g/L or of 0.1 g/L, the APFO could no longer be reduced below LC/MS/MS tolerance (0.46 mM), despite 16 h evaporation. Addition of 50 mM HCl to protein-containing sample greatly improved the efficiency of APFO removal (Fig. 2.11(B)). However, the level of APFO remaining in the 1 g/L BSA sample was higher than the threshold tolerance. In a 0.1 g/L BSA sample, efficient removal of APFO was achieved. Thus, a single-step evaporation protocol is sufficient to reduce the concentration of APFO to a level permitting subsequent LC/MS analysis. However, the composition of the sample, including the presence of (non-volatile) ionic species and protein concentration, has significant influence on the rate of evaporation. These variables should be taken into consideration if APFO (or PFOA) is to be incorporated in a proteomic workflow. Nonetheless, this one-step removal strategy justifies incorporation of APFO in the proteome workflow. As such, APFO can be recommended as a suitable alternative to SDS.

2.4. Conclusions

The fluorinated surfactant perfluorooctanoic acid (PFOA) is viewed as a suitable alternative to SDS for proteome processing; however, such a classification is made with caution. First, the form of the surfactant (protonated or salt form) must be considered, with

preference to the ammonium salt for proteome solubilization (*i.e.* APFO). Second, while the fluorosurfactants are more tolerable to both direct infusion ESI-MS and LC/MS, the improved tolerance is only marginally greater than SDS (~2-fold higher, on a molar basis). Thus, APFO must be removed from the sample prior to bottom-up (or top- down) MS analysis. While considerable methods are available for SDS removal, the simplicity of a one-step evaporation protocol reported here for APFO removal provides a facile alternative to SDS removal. The evaporation strategy is perhaps most appealing where protein loss is of concern. The evaporation efficiency is maximized in acidic medium, and with low non-volatile salt content. Incorporation of an organic solvent precipitation early in the workflow would ensure removal of salts from the sample, as APFO has been demonstrated as an effective strategy for resolubilization of the protein pellet.

Chapter 3

Assessment of membrane protein recovery following precipitation with organic solvents [2]

3.1 Introduction

Prior to MS analysis, membrane proteins are typically solubilized in detergent and fractionated using methods such as gel electrophoresis, or through various forms of chromatography. Preferably, the detergents should be present throughout the separation in order to maintain protein solubility during fractionation. However, separation methods may be incompatible with detergents as, for example, small amount of the anionic detergent SDS will harm ion exchange chromatography by altering the charge of the proteins. SDS is the most powerful detergent for solubilization of membrane proteins. [17] Unfortunately, above a critical concentration of 0.01%, SDS [29] will suppress ESI MS signals for proteins and peptides. [31] Surfactants are also detrimental to RPLC which is integral to the LC/MS experiment. [78] As a result, MS analysis of membrane proteins remains a challenging task. However, SDS may still be incorporated into a proteomics workflow, so long as the surfactant is removed prior to LC/MS analysis.

Currently, there are several methods for detergent removal, including dialysis, [100] strong cation exchange, [78] spin columns, [101] ultracentrifugation devices, [80] gel filtration, [35] well as precipitation with organic solvents. [36, 76] These methods vary in terms of their ability to purify the protein, with levels spanning from 10-to over 1000-fold reduction of the surfactant. Given the tolerance of LC/MS towards SDS (<0.01%), any useful approach

[2] A version of this chapter has been prepared to be submitted. Dennis Orton contributed to the generation of Figure 3.8.

to purify the protein must achieve this level of reduction of the detergent. Our lab has previously demonstrated the effectiveness of solvent precipitation (acetone/CMW) to achieve this level of SDS reduction. [29] Although these solvent precipitation methods are efficient strategies to remove SDS, the recovery of protein is also an important consideration of the method.

The high recovery of proteins following solvent precipitation is an important consideration prior to LC/MS analysis. Thongboonkerd *et al.* showed that acetone tends to precipitate more acidic urinary proteins, while some basic proteins are often absent. [102] Barritault *et al.* acquired 80% ribosomal protein recovery following cold acetone SDS removal protocol for samples initially prepared in 0.5% SDS. [103] Puchades *et al.* reported the recovery of two standard proteins (myoglobin and cytochrome c) following SDS removal with CMW or acetone. [76] They determined protein recovery was "only" 50% with CMW and 80% with acetone. Most recently, Crowell *et al.* were able to maximize the recovery of water soluble proteins in acetone (*i.e.* approaching 100% yield for proteome mixtures) by adding an ion pairing reagent (*e.g.* NaCl) to neutralize the charge of the protein. [104] In the latter report, the authors demonstrated the importance of the ionic strength as a controlling role in the precipitation efficiency of water soluble proteins. Indeed, this is an efficient method to precipitate proteins while achieving the necessary purity (in terms of SDS reduction) to enable LC/MS analysis.

Although the level of protein recovery through solvent precipitation has been reported, to date there is no such quantitation for complex membrane protein mixtures. Researchers have struggled to show an efficient recovery (approaching 100%) of membrane proteins following precipitation with organic solvents. Thongboonkerd *et al.*

determined a preference towards recovery of hydrophilic urinary proteins when subject to acetone precipitation. [102] Membrane protein recovery through precipitation is further challenged by the task of re-solubilizing the protein pellet in an MS-compatible solvent system. The re-solubilization step (*i.e.* solubilization of proteins following precipitation) prior to LC/MS analysis is essential in order to achieve reliable separation and/or MS analysis.

Following precipitation, membrane proteins will require appropriate solvent additives to improve solubilization. It should also be noted that these additives must be compatible with LC/MS. The inclusion of trypsin to digest a precipitated sample is an effective strategy to achieve high protein recovery. [105] However, if the protein integrity is required, as seen for top-down proteomics, enzymes must be avoided in favour of other strategies. Low concentrations of acetonitrile (*e.g.* 5%) and formic acid (*e.g.* 0.1%) have been applied to re-solubilize peptides prior to mass spectrometry. [106] Although commonly used to solubilize peptides, this solvent (5% acetonitrile/0.1% formic acid) has a low efficiency to dissolve intact proteins. Other approaches have been attempted to solubilize a cellular membrane protein fraction, including the use of chaotropes (*e.g.* 8 M urea), MS-compatible surfactants (*e.g.* Rapigest from Waters or PPS Silent from Protein Discovery) and organic solvents. Unfortunately these methods demonstrate little improvement over a proteomics workflow that incorporates SDS. [70, 107] High levels of organic acid (*e.g.* formic acid and acetic acid) have been demonstrated to efficiently dissolve proteins, [108] including membrane proteins, [32, 109] and they have been incorporated in the proteomic workflow. The use of high levels of the formic acid for resolubilization of the protein pellet following detergent removal (*e.g.* acetone and CMW approaches), has yet to be demonstrated.

Beyond compatibility with chromatography and mass spectrometry methods, this strategy is able to maintain the membrane proteins in solution prior to LC/UV and/or LC/MS analysis.

In this study, protein precipitation methods optimized for SDS removal (acetone and CMW) are applied to recover membrane proteins isolated from *E. coli*. We demonstrate high recovery of membrane proteins, as revealed through LC/MS/MS analysis of the resulting pellet and supernatant, where the majority was observed in the pellet. To date, the assessment of membrane protein recovery following precipitation with the solvents cited above has not been performed. In addition, we also establish an effective method for protein resolubilization following protein precipitation. It was found that 80% formic acid is effective to quantitatively recover proteins in the pellet, and is also compatible with the LC and/or MS experiments.

3.2 Experimental

3.2.1 Materials

Myoglobin from equine heart (Cat. # 100684-32-0), TPCK-treated trypsin (Cat. # T8802), and lysozyme from chicken egg white (Cat. # L-6876) were purchased from Sigma Aldrich (Oakville, Canada), while SDS (Cat. # 161 0302) and all remaining reagents for SDS-PAGE were obtained from BioRad (Mississauga, Canada). Acetone (Cat. # 320110), chloroform (Cat. # 67-66-3), methanol (Cat. # 67-56-1) and solvents of HPLC grade were from Fisher Scientific (Ottawa, Canada), with Milli-Q water purified to 18.2 MΩ cm.

3.2.2 *E. coli* total and membrane proteome extraction

E. coli total proteins were harvested and extracted according to QIAGEN protocols (QIAGEN Manual for Good Microbiological Practices, Mississauga, Canada). A single

colony of *E. coli* from Luria-Bertani (LB) agar plate was inoculated into the LB broth media (Sigma- Cat # L3022). Incubation was performed at 37 °C with shaking for 16 hrs until the OD_{600} reads approximately 1. After centrifugation, the bacterial pellet was collected and store at -20 °C until just prior to use (one day).

E. coli membrane proteins were harvested and extracted according to Wu *et al.* 2011 [72] with some modifications. Resuspension of the cells was made in 30 mL water with 3 mL of protease inhibitor cocktail (Sigma- Cat # 2714). The cells were then twice passed through a French press [3] (Aminco, Rochester, NY) at 10,000 psi. The lysate was centrifuged at 2300 × g for 15 min at 4°C to remove unbroken cells. *E. coli* membrane proteins were collected by two consecutive rounds of ultracentrifugation (Beckman Coulter optima Le-80K, Mississauga, CA). The ultracentrifugation was performed at 118,000 × g (55 min at 4°C) while a second spin was executed at 166,811 × g (40 min at 4°C). Between each centrifugation step, the supernatant was discarded and the pellet was resuspended in 50 mM Tris-HCl. The pellet was stored at -20 °C until just prior to use.

3.2.3 Protein precipitation

E. coli membrane or total pellet were resuspended in 1% SDS (100 µL) at 95 °C for 5 min followed by 30 minutes sonication prior to protein precipitation. Samples were precipitated overnight at -20 °C following the addition of acetone in a 4:1 ratio over the sample. Samples were centrifuged for 10 minutes at 21000 × g (top layer or supernatant A was saved for further analysis). Additional washing of the pellet (two wash cycles) was executed using 400 µL aliquots of cold acetone, with immediate centrifugation at 21000 ×

[3] I would like to thank the NRC (National Research Council) for the French Press used to lysate *E. coli* cells.

g for 10 min without mixing before removing the acetone layer (or supernatant B that was saved for further analysis). The supernatants A and B were combined and evaporated with a Savant SpeedVac concentrator. The protein pellet was stored at -20 °C prior to use. For SDS PAGE (see Section 3.2.6), pellet and combined supernatants fractions were resuspended in Laemmli buffer (50 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 0.1% *w/v* bromophenol blue). [110]

For chloroform/methanol/water precipitation, the protocol is an adaptation of the method utilized by Wessel and Flugge. [36] In brief, 400 µL of methanol, followed by 100 µL chloroform, then 300 µL of water was added to 100 µL of sample, with brief vortexing after each solvent addition. The sample was centrifuged for 10 min for 21000 × g and the top layer (*i.e.* methanol fraction) was removed and saved to further analysis (supernatant I). A 400 µL aliquot of methanol was added with gentle mixing to encourage the solvents to mix. The vial was centrifuged for 10 min at 21000 × g and the supernatant (supernatant II) was saved to further analysis. The resulting protein pellet was washed once with 400 µL of methanol, with an additional 10 min centrifugation (supernatant III was saved for further analysis). Also, supernatant I, II and III were collected to the same vial. For SDS-PAGE separation (Section 3.2.6), the combined supernatants were evaporated to dryness with a Savant SpeedVac concentrator.

3.2.4 Exosome proteins analysis

Urinary exosome proteins were extracted according to Lamparski *et al.* [111] A 150 mL urine sample was acquired from a healthy donor and stored at -20 °C prior to use (two days). The thawed urine sample was centrifuged at 17000 × g for 10 min at 4 °C. The supernatant was collected and placed onto a 30% sucrose layer (~600 µL of sucrose

solution in 20 mM HEPES buffer). Following 1 hr centrifugation at 200,000 × g (4 °C), 550 µL of the sucrose cushion/ interface was collected and transferred to a new tube in PBS/protease inhibitor (200 µM AEBSF, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, 30 nM aprotinin, 13 µM bestatin, 100 µM EDTA (ethylenediaminetetraacetic acid), 1.4 µM E-64, 100 nM leupeptin). A second centrifugation at 200,000 g (4 °C) for 1 hr was then performed. The supernatant was removed (~200 µL) remaining and the pellet was resuspended in PBS/protease inhibitor (as described above).

Exosome proteins (10 µg/ 100 µL in 1% SDS/PBS/protease inhibitor as described above) were precipitated with CMW or precipitated overnight at -20 °C following the addition of acetone (four times the sample volume) as described in Section 3.2.3 above. For desalting and volume reduction, controls (samples before precipitation) were concentrated through a 3 kDa MWCO (molecular weight cut off) filter (Cat # L 42404) from Amicon Microcon (Milipore, Bedford, MA, USA), according to the manufacturer's instructions.

3.2.5 BCA quantification assay

Protein concentration was determined through a BCA protein assay according to the manufacturer (Thermo Scientific). Protein quantification was relative to a calibration curve comprising bovine serum albumin (BSA) over the range 0.5 µg to 2.5 µg. Briefly, the calibration standards and protein samples were incubated at 60 °C, in the presence of BCA working reagent, for approximately 20 min. Then, the samples were incubated for an additional 20 min at room temperature, and then the absorbance was measured at 562 nm using an Agilent 8353 spectrophotometer (Palo Alto, CA) with a 1-cm quartz cuvette.

3.2.6 SDS-PAGE and Western blot analysis

SDS-PAGE was executed as previously described. [29] For visualization of the *E. coli* membrane or total proteins, the pellet and supernatant (see Section 3.2.3 above) were mixed with 40 µL (for samples with 100 µg/ 100 µL protein concentration) or 20 µL Laemmli buffer, [110] then heated at 95 °C for 5 min and briefly centrifuged at 21,000 × g. Twenty microliters was then loaded onto a 1 mm, 12% polyacrylamide gel and run at 30 mA constant current until the dye front reached the lower edge of the gel. Proteins bands were stained by Coomassie. For exosome proteins, Western blot analysis was executed according to Burnette, [112] and proteins were blotted using Alix antibodies (Novus Biologicals Inc., Littleton, USA)

3.2.7 In-gel trypsin digestion and sample clean up

For in-gel trypsin digestion, the control (*i.e. E. coli* proteins before precipitation), pellet or supernatant (after evaporation) layers were mixed with 20 µL Laemmli buffer and the SDS-PAGE conditions were performed as described in the section above. Each condition (control/pellet/supernatant) was loaded in triplicate (one gel for acetone and another for CMW). For bottom-up analysis, the entire gel was subject to 3 × 10 min washes in water to reduce the level of SDS in the gel. Ten identically sized bands were manually excised per lane (30 bands total per condition – 90 bands per gel), placed in separated vials, and subjected to in-gel digestion with trypsin using the method of Shevchenko *et al.* [66]

Following in-gel trypsin digestion, the peptides were subjected to automated sample cleanup using reversed-phase chromatography (0.5 × 5 mm, 5 µm C_{18} beads) on an Agilent 1200 HPLC system with UV detection at 214 nm. The desalted peptides were collected as a single fraction and the solvent was completely removed in a Savant

SpeedVac concentrator before the dried peptide sample was stored at -20 °C until just prior to LC-MS/MS analysis.

3.2.8 LC/UV analysis of intact proteins

Following the protein precipitation, the *E. coli* membrane protein pellets were resuspended with 100 µL using one of three solvent systems: 5% acetonitrile/0.1% formic acid, 8M urea, or 80% formic acid. Each sample was subjected to repeat pipetting up and down in a pipette tip to aid resuspension of the pellet, followed by vortexing for 1 min, and 30 min sonication. The urea sample was not subjected to sonication due to the heat buildup that occurs during this step. Resuspended samples were subjected to LC separation on a 10 mm × 0.5 mm self-packed POROS R2 column (20 µm beads; Applied Biosystems, Mississauga, Canada). An Agilent 1200 HPLC system was used with UV detection at 214 nm. The flow rate was 100 µL/min. Solvent A was water plus 0.1% trifluoroacetic acid (TFA) and solvent B was acetonitrile with 0.1% TFA. The gradient was initially set to 5% B and held for 32 min to allow unretained components to flush through, then adjusted as follows: jump to 85% B at 32.01 min, hold for 5 min and then drop to 5% B at 37.01 min. To avoid any cross contamination, a wash step was also added during the run by setting to 95% B at 57.01 min, hold for 1 min and then drop to 5% B at 58.02 min. In addition to the solvent gradient, a temperature program was also applied (Agilent 1200 HPLC system equipped with column thermostat). The column was initially held at 25°C, and the temperature was increased to 80°C by 16 min and held for 24 min until elution of *E. coli* membrane proteins was observed, then dropped to 25°C at 50.01 min. The total run time was 92 min. Results reported here are from averaging the intensities obtained from five replicates.

3.2.9 LC-MS/MS, bottom-up analysis and data searching

Cleaned peptides were mixed with 5% acetonitrile/0.1% formic acid (corresponding to around 1 μg total protein per sample) was then subjected to LC-MS/MS in four replicates. The column was a self-packed 20 cm × 75 μm spray tip (New Objective, Woburn, MA) using 4 μm C_{12} beads (Phenomenex, Torrance, CA). The flow rate was 0.25 μL/ min. Solvent A was water with 0.1% formic acid and solvent B was acetonitrile with 0.1% formic acid. The gradient was initially set to 5% B. The % B was increased to 7.5%, 20%, 25%, 35%, and 80% by 0.10, 45, 57.5, 60 and 61 min, respectively. Then, the % B was held at 80% for 3.9 min and lowered to 5% by 65 min. MS was through nanospray ionization on an LTQ linear ion trap instrument (Thermo Fisher Scientific, Waltham, MA) employing a 'triple play' data dependent scan. 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. Charge state screening was enabled to ignore singly charged ions, ions with a charge of 4 and greater, or ions where the charge state could not be assigned. Data were searched using the Proteome Discoverer 1.3 software from Thermo, which uses the SEQUEST search engine.

3.2.10 Direct infusion of ESI-MS analysis of intact proteins

Standard protein (myoglobin) was re-suspended with 80% formic acid, before and after acetone precipitation, and then infused to ESI-MS analysis at a flow rate of 20 μL/ min using a syringe pump. A 10 μL sample (5 μg total per injection) was injected into an LCQ duo ion trap mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA) equipped with electrospray ionization source. The myoglobin peaks were monitored using the following MS operating parameters: Spray voltage: 4 kV; capillary temperature: 250

°C; capillary voltage: -38 V; tube lens offset: 25 V; number of microscans: 3; maximum inject time: 200 ms; scan range: *m/z* 900 to 1500.

3.2.11 Data analysis

Intact protein quantification through LC/UV was performed according to Orton *et al.* by exporting the chromatographic traces and integrating peak areas with Microsoft Excel. [113] For MS quantification of the common membrane proteins identified in the pellet and supernatant, the peptide spectrum match (PSM) was obtained for each replicate of the protein identified. [114]

3.3 Results and Discussion

Proper sample preparation is essential for obtaining reliable results in proteomic experiments. Protein precipitation with organic solvents such as acetone and chloroform/methanol/water (CMW) is effective for sample purification, including the elimination of SDS. [29] The recovery of water-soluble proteins has been determined for acetone precipitation. [104] However, these results may not extend to hydrophobic (membrane) proteins which may show increased solubility in an organic solvent system.

3.3.1 Efficiency of solvent precipitation for membrane proteins

A membrane proteome extract from *E. coli* was chosen as a representative proteome to evaluate recovery efficiency with acetone and CMW precipitation. A 100 µg sample (in 100 µL) was prepared in the presence of 1% SDS and subjected to solvent precipitation. Figure 3.1 shows the *E. coli* membrane protein recovery following acetone or CMW precipitation. Analysis of proteins from both the pellet and the supernatant fraction by SDS-PAGE demonstrate very weak protein bands in the acetone and CMW fractions (Figure 3.1A & 3.1B, respectively). The protein bands in the pellet were comparable to the control

(non-precipitated membrane proteins). The gel images are therefore consistent with high protein recovery for both the acetone and CMW precipitated membrane proteins. As a quantitative measure of protein recovery, a BCA assay was applied to the pellet and supernatant fractions. The acetone pellet contained approximately 92% of the *E. coli* membrane proteins (Figure 3.1C) while 86% recovery was observed in the CMW pellet (Figure 3.1D). A small fraction of the membrane proteins was detected in the supernatant (1% from acetone; 13% for CMW), accounting for the total protein material in the sample.

Previous studies have shown that precipitation of proteins with organic solvents may be affected by the protein concentration. [76] It is therefore important to quantify recovery as a function of the initial protein concentration. *E. coli* membrane proteins were also prepared at 10 μg/ 100 μL, which is 10-fold more dilute than was shown in Figure 3.1. As seen in Figure 3.2, similar recovery was obtained for the more dilute protein sample. Protein recovery following acetone precipitation was measured around 95% and as 79% in CMW.

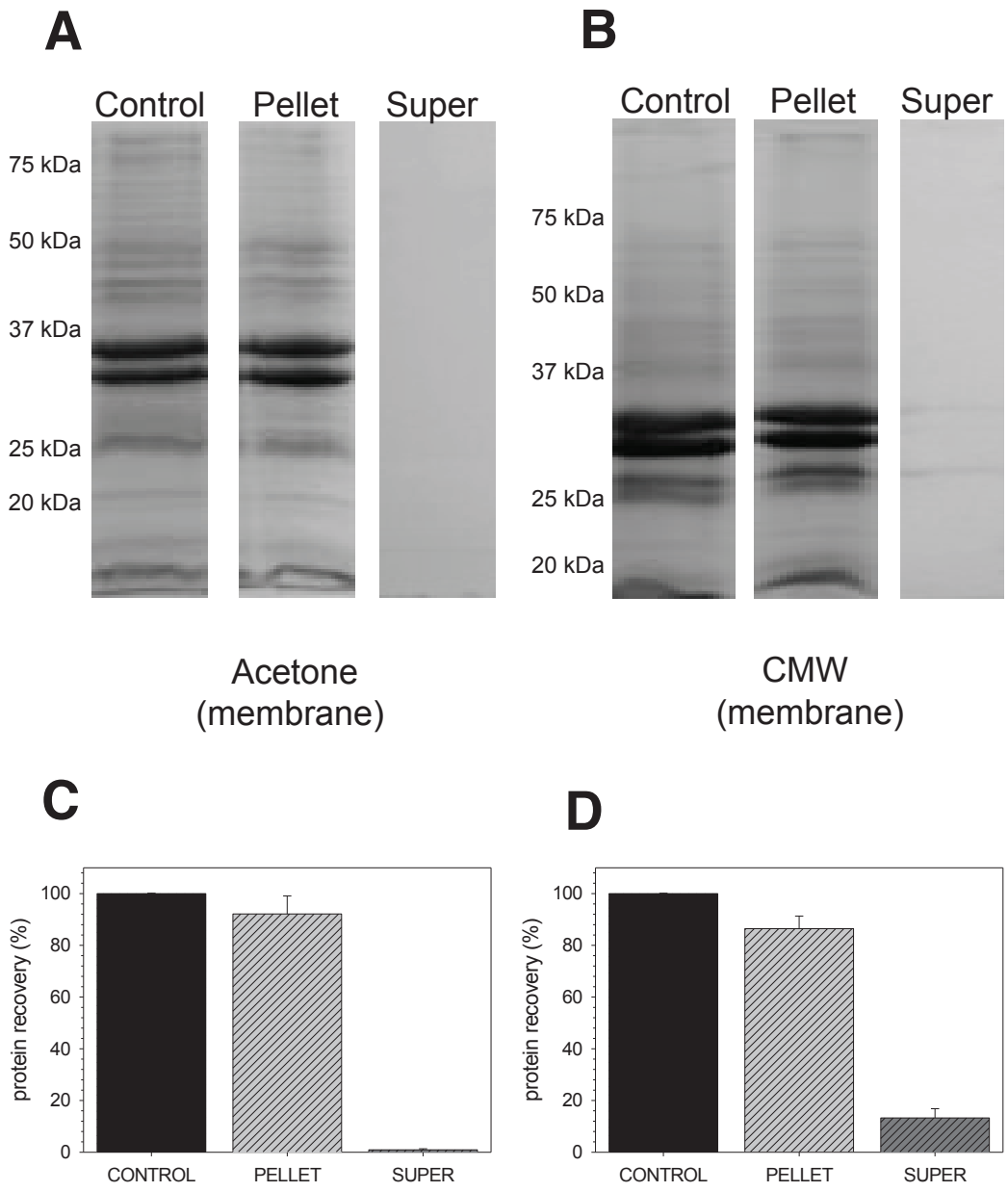

Figure 3.1 Protein recovery from the pellet and supernatant of the *E. coli* membrane proteome following protein precipitation with acetone and chloroform/methanol/water (CMW). SDS-PAGE images of fractions collected from *E. coli* membrane proteins after (A) acetone or (B) CMW precipitation. A total of 100 µg *E. coli* membrane proteins in 100 µL of 1% SDS were precipitated with these organic solvents. For the BCA assay (C & D), proteins were re-suspended in 1% SDS.

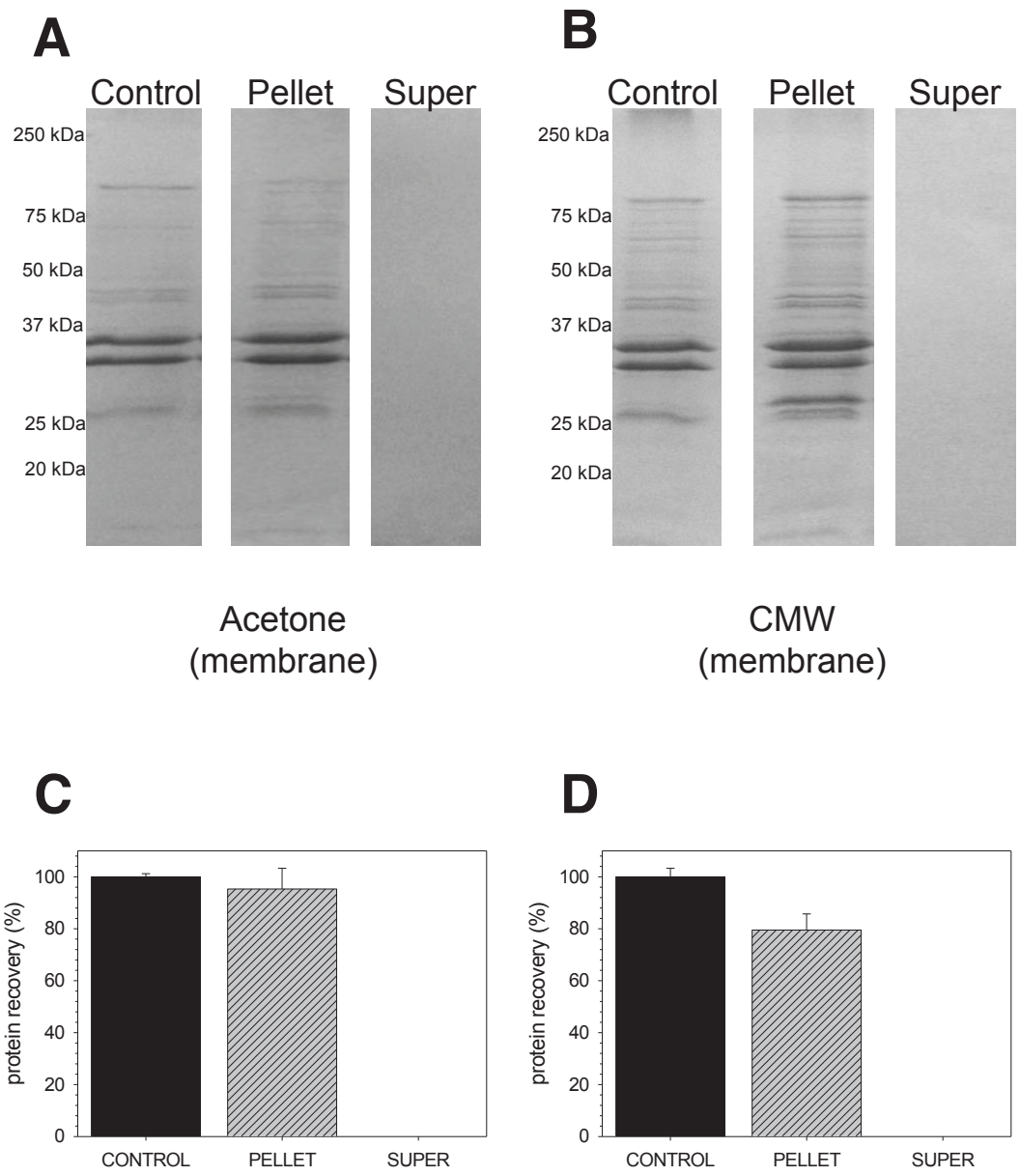

Figure 3.2 Protein recovery from the pellet and supernatant of the *E. coli* membrane proteome following protein precipitation with acetone and CMW. A total of 10 µg/ 100 µL *E. coli* membrane proteins in 1% SDS were precipitated with these organic solvents. For SDS-PAGE, the re-suspension of pellet and supernatant was performed in presence of 20 µL of Laemmli buffer, and then loaded all volume (*i.e.* 20 µL) into SDS-PAGE. For the BCA assay (C & D), proteins were re-suspended in 1% SDS.

Similar outcomes were observed with *E. coli* total proteome fraction (including soluble as well as membrane proteins). Figure 3.3 illustrates the recovery of *E. coli* total

proteins after acetone and CMW precipitation methods. SDS-PAGE demonstrated a complete absence of *E. coli* proteins in the supernatant fractions of acetone (Figure 3.3A) or CMW (Figure 3.3B). In other words, the majority of *E. coli* total proteins precipitate and are recovered in the pellet with acetone and CMW solvents. The BCA assay was able to detect a small amount of *E. coli* total proteins in the supernatant of acetone (~1.6%) and CMW (1.5%), as seen in Figure 3.3C and Figure 3.3D, respectively. Given the amount detected through BCA, and considering the lower limit of visualization with Coomassie (~1 μg total protein, results not shown), one would have expected to observe faint protein bands in the supernatant fractions. Similarly, the proteins bands shown in the membrane supernatant fractions (Figure 3.1) are also weaker than would have been expected. These results are therefore somewhat contradictory to the BCA quantitation assay.

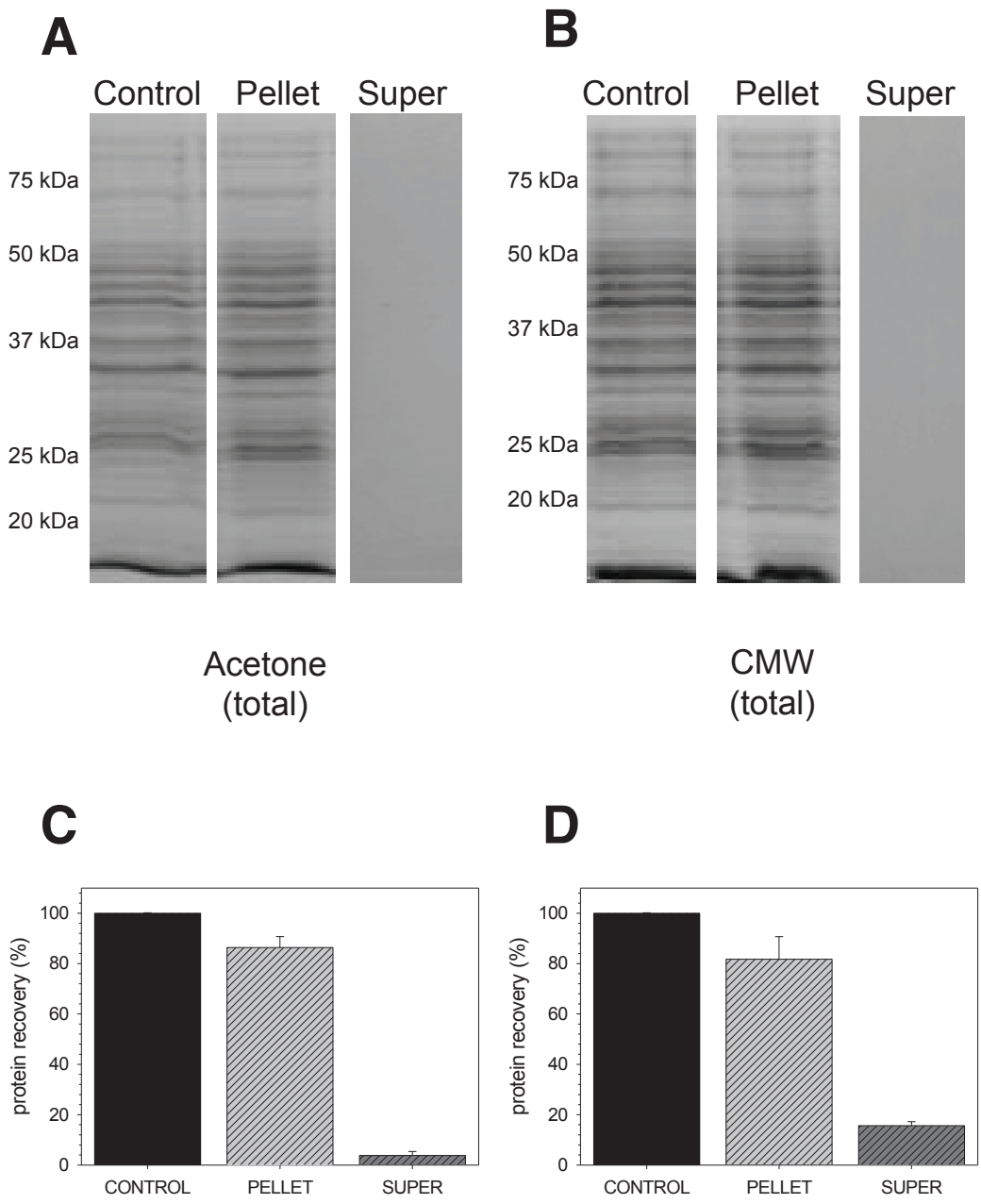

Figure 3.3 The protein recovery of the *E. coli* total (whole) proteome following protein precipitation with acetone and CMW. A total of 100 µg/ 100 µL *E. coli* total proteins in 1% SDS were precipitated with these organic solvents. For SDS-PAGE, the protein pellet and supernatant after acetone and CMW was re-suspended in Laemmli Buffer. For the BCA assay (C & D), proteins were re-suspended in 1% SDS.

The BCA assay has been widely utilized to quantify proteins samples. The principle of this method is that the bicinchoninic acid can detect the reduction of copper by protein

in an alkaline solution. [115] Compared to Coomassie dye-binding methods, the advantage of the BCA assay is that the peptide backbone also contributes to color formation [116] However, this BCA quantification assay presents some disadvantages in that it is susceptible to interference by chemicals that might be present in the protein sample, including reducing agents (*e.g.* dithiothreitol, DTT, and beta-mercaptoethanol), copper chelators (*e.g.* EDTA), and highly concentrated buffers. [116] Smaller peptide fragments, as well as free amino acids, will also give rise to detectable signals with BCA, though these components would not be observed in an SDS-PAGE gel. Given the disagreement between BCA and SDS-PAGE, the efficiency of acetone and CMW precipitation protocols were quantified through an independent method, namely Western blot analysis.

Figure 3.4 illustrates the Western blot analysis of exosomal membrane proteins using the Alix antibody, [117] following acetone and CMW precipitation. Alix is a protein marker utilized for exosome vesicles. A control (non-precipitated sample) was analyzed alongside the pellet and supernatant. As seen in this figure, no protein band was observed in the supernatant fraction, while the intensity of the protein band in the pellet was similar to that of the control. Furthermore, this Western blot analysis showed multiple bands in the acetone pellet compared to control and/or CMW pellet. Acetone modification of peptides is well-known in the literature, [118] but, up to now, there is no evidence of protein degradation following acetone precipitation. Although the results presented in the Figure 3.4 indicate the protein degradation following acetone precipitation, more studies still need to be done in order to conclude this hypothesis. Western blot analysis is based on a single membrane protein, and therefore to better understand the efficiency of these acetone and

CMW protocols to precipitate membrane proteins as a whole, it is necessary to observe a complex mixture.

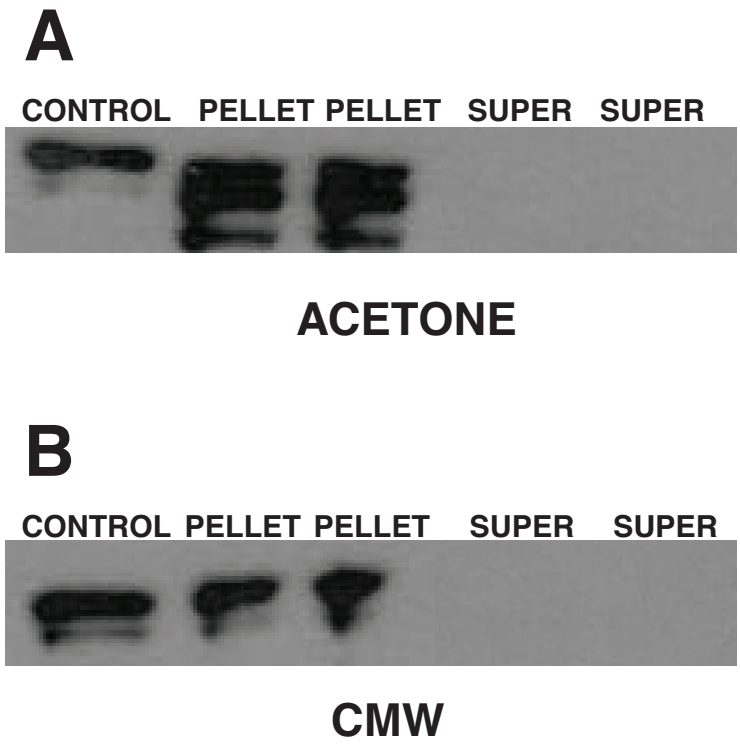

Figure 3.4 *E. coli* membrane proteins blotted against Alix antibody. STD represents the control sample (before precipitation), while super stands for supernatant. 10 µg/ 100 µL of *E. coli* membrane was precipitated with (A) acetone or (B) chloroform/ methanol/ water (CMW).

3.3.2 Efficiency of protein precipitation through LC/MS analysis

To test acetone and CMW precipitation for a more complex membrane protein mixture, the precipitated *E. coli* membrane proteins were subjected to LC/MS analysis. Figure 3.5 summarizes the number of proteins identified using a typical proteome workflow strategy (bottom-up). [119] Figure 3.6 summarizes the peptide information from the same samples. A few key points can be made. First, comparison of the precipitation methods reveals a slightly larger number of *E. coli* membrane proteins identified from the

acetone pellet over the CMW pellet (289 *vs* 280 respectively). However, more *E. coli* membrane proteins were identified in the acetone supernatant (28 proteins, Figure 3.5A), than the CMW supernatant (7 proteins, Figure 3.5B). Also, Figure 3.5 illustrates the number of *E. coli* membrane proteins identified without precipitation (control samples). This is consistent with the observation of higher protein yield through acetone precipitation. Jiang *et al.* [120] also reported that precipitation of human plasma proteins with acetone resulted in a more concentrated sample than CMW method. As a result of slight difference between these techniques, the method of choice may be based on the type of experiment performed. Each method provides a distinct advantage: acetone precipitation is technically simpler to perform, while the CMW protocol is much quicker to complete.

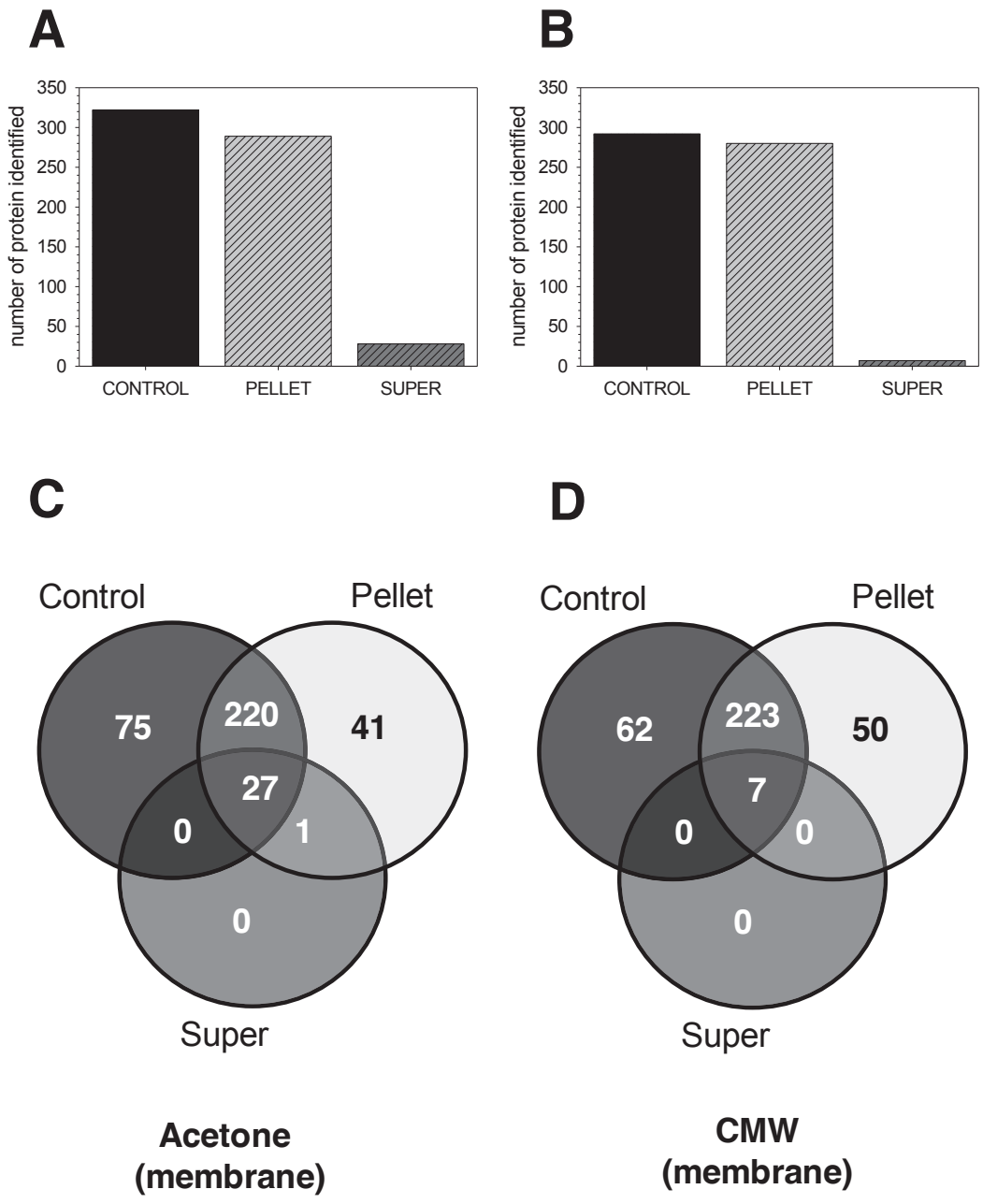

Figure 3.5 Venn diagrams showing the number of *E. coli* membrane proteins identified by mass spectrometry after employing protein precipitation with acetone and CMW. "Super" represents supernatant.

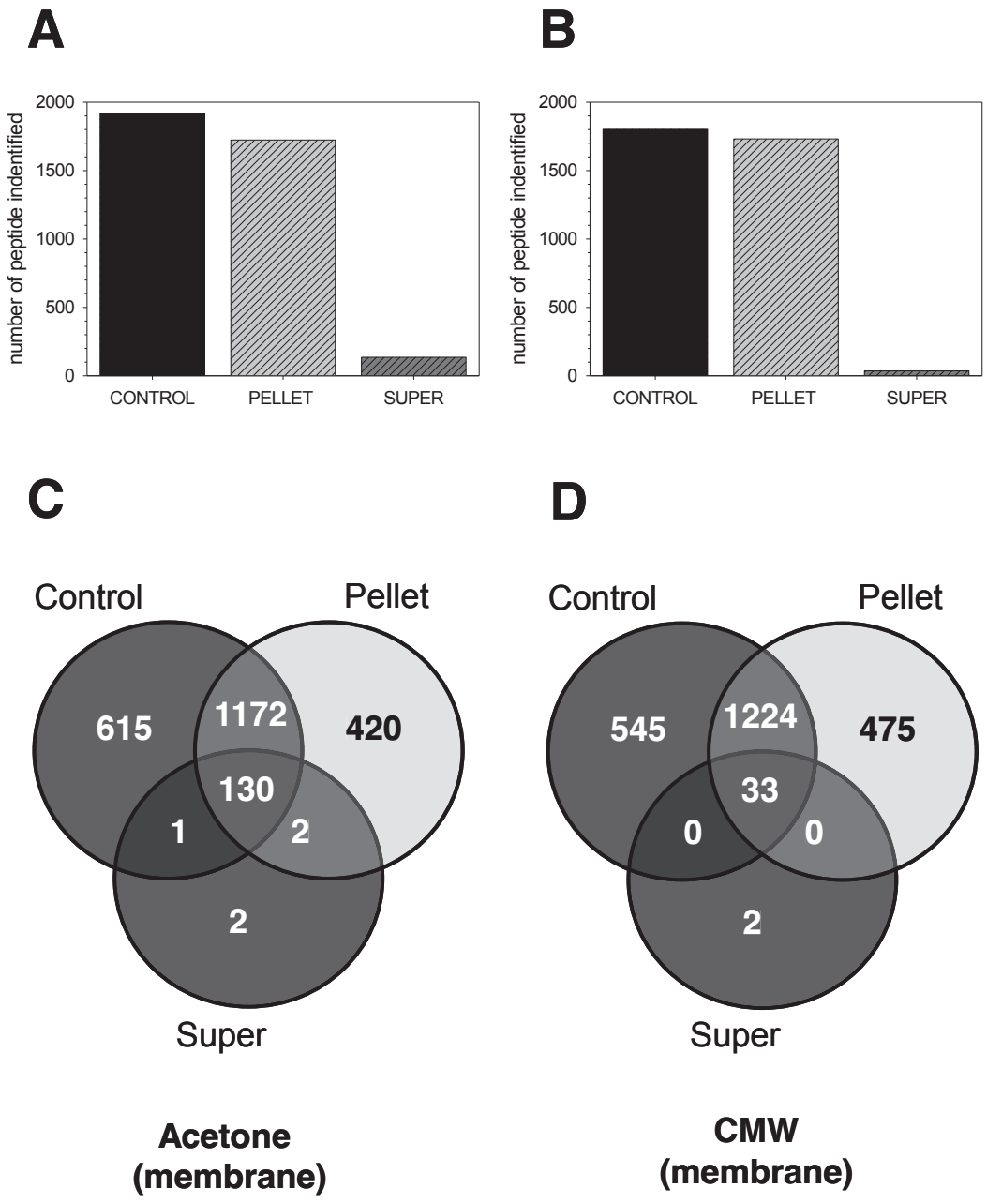

Figure 3.6 Numbers of *E. coli* membrane peptides identified by mass spectrometry after employing protein precipitation with acetone and CMW. Venn diagrams displays the number of *E. coli* membrane peptides identified *via* acetone or CMW precipitation methods. "Super" represents supernatant.

Comparing the number of proteins (peptides) identified from the pellet *vs* supernatant, one can obtain a measure of protein precipitation efficiency. Approximately

10 times as many proteins were detected in the acetone pellet (289 *vs* 28 membrane proteins in the supernatant, Fig 3.5C). It is important to note, however, that all 28 proteins identified in the supernatant were also identified in the pellet (Table 3.1). No protein was uniquely detected in the supernatant fraction. Likewise, of the 7 proteins identified in the supernatant from CMW, all of these proteins were also seen in the pellet fraction (Table 3.2). Previous studies demonstrated that acetone precipitation has a preference toward the loss of hydrophobic proteins. [102, 121] However, these authors used 50% acetone for recovering proteins, a non-ideal condition for efficient protein precipitation. [104] Puchades *et al.* showed 80% and 50% recovery of two standard proteins (myoglobin and cytochrome c) with acetone and CMW precipitation, respectively. [76] However, their outcomes are based on visual comparison of protein band intensity within SDS-PAGE. Throughout MS measurements, our data suggest that acetone and CMW precipitation are extremely efficient to precipitate hydrophobic (membrane) proteins.

A quantitative analysis was performed on the proteins identified in the supernatant, relative to those observed in the pellet. Table 3.1 and 3.2 summarizes the *E. coli* membrane proteins simultaneously identified in the pellet and supernatant fractions of acetone and CMW precipitations, respectively. These tables include the peptide spectrum match (PSM). This algorithm, PSM, assigns a numerical value to a peptide-spectrum. [114] The PSM value is frequently associated with concentration of the protein identified. In other words, a larger value of PSM corresponds to a greater concentration of the particular protein identified. The ratio, which was calculated by dividing the pellet PSM over supernatant PSM, represents a measure of relative protein abundance in the two fractions. With few exceptions, the majority of proteins were of greater abundance in the pellet (Table 3.1 and

3.2). Similar outcomes are also observed with *E. coli* total proteins precipitate with acetone (Table 3.3) and CMW (Table 3.4). In other words, these tables indicates that even though some proteins were detected in the supernatant fraction of the acetone and CMW precipitations, the majority of the given protein was partitioned to the pellet fraction. As a consequence, qualitative MS analysis of the pellet alone will not be affected by the precipitation process.

Table 3.1 Relative abundance of *E. coli* membrane proteins identified in both the pellet and supernatant after employing protein precipitation with acetone.

protein name	accession	PSM (pellet)	PSM (super)	ratio
ATP synthase subunit alpha	C9QXA2	104	3	35
ATP synthase subunit b	C9QXA0	138	4	35
Outer membrane protein assembly factor BamA	C9QRL1	84	3	28
Succinate dehydrogenase flavoprotein subunit	C9R0P7	82	3	27
Peptidoglycan-associated lipoprotein	C9R0M9	48	3	16
Peptidyl-prolyl cis-trans isomerase	C9QQ77	79	5	16
Protein translocase subunit SecD	C9QQB0	72	5	14
LPS-assembly protein lptD	C9QS98	42	3	14
FecA protein	C9QTE7	34	3	11
60 kDa chaperonin	C9QU57	43	4	11
Lipoprotein	C9QRJ1	32	3	11
Outer membrane protease	C9R1F9	89	9	10
OmpX	C9R037	54	6	9
ATP synthase subunit beta	C9QXA4	69	8	9
Tryptophanase	C9QXC8	51	6	9
Outer membrane channel protein	C9QYX6	69	9	8
Pectinesterase	C9R0K5	27	4	7
Outer membrane porin protein C	C9QR92	265	42	6
Elongation factor Tu 2	C9QV97	153	25	6
Long-chain fatty acid outer membrane transporter	C9QQJ2	30	5	6
50S ribosomal protein L1	C9QV93	16	3	5
Outer membrane assembly lipoprotein YfgL	C9QPR1	21	4	5
Outer membrane protein F	C9QZE7	134	28	5
Maltoporin	C9QV41	17	4	4
OmpA domain protein transmembrane	C9QZB9	690	178	4
LPP repeat-containing protein	C9QU30	45	42	1
D-ribose transporter subunit RbsB	C9QX85	4	5	0.8
Thioredoxin	C9QX67	14	27	0.5

PSM: peptide spectrum matching; super: supernatant; ratio: PSM (pellet)/ PSM (super)

Table 3.2 Relative abundance of *E. coli* membrane proteins identified in both the pellet and supernatant after employing chloroform/methanol/water protein precipitation.

protein name	accession	PSM (pellet)	PSM (super)	ratio
Elongation factor Tu 2	C9QV97	203	4	50
Outer membrane porin protein C	C9QR92	218	12	18
Outer membrane protein F	C9QZE7	113	10	11
OmpA domain protein transmembrane	C9QZB9	592	90	6
Thioredoxin	C9QX67	27	18	1
LPP repeat-containing protein	C9QU30	41	33	1
D-ribose transporter subunit RbsB	C9QX85	3	6	0.5

PSM: peptide spectrum matching; super: supernatant; ratio: PSM (pellet)/ PSM (super)

Table 3.3 Relative abundance of *E. coli* total proteins identified in both the pellet and supernatant after employing acetone precipitation.

protein name	accession	PSM (pellet)	PSM (super)	ratio
D-ribose transporter subunit RbsB	C9QX85	73	1	73
Outer membrane protein F	C9QZE7	42	2	21
Elongation factor Tu 2	C9QV97	45.5	3	15
OmpX	C9R037	7.5	1	7.5
LPP repeat-containing protein	C9QU30	5.5	2	2
OmpA domain protein transmembrane	C9QZB9	22	12	1
Outer membrane porin protein C	C9QR92	2.5	8	0.31

PSM: peptide spectrum matching; super: supernatant; ratio: PSM (pellet)/ PSM (super)

Table 3.4 Relative abundance of *E. coli* total proteins identified in both the pellet and supernatant after employing CMW precipitation.

protein name	accession	PSM (pellet)	PSM (super)	ratio
60 kDa chaperonin	C9QU57	42	1	42
Glyceraldehyde-3-phosphate dehydrogenase A	C9QTS9	24	1	24
Phosphoglycerate kinase	C9QZJ1	20	1	20
Alpha-galactosidase, NAD (P)-binding	C9QU80	17.5	1	17.5
Elongation factor Tu	C9QV97	48.5	3	16
Outer membrane protein F	C9QZE7	46	3	15
D-ribose transporter subunit RbsB	C9QX85	87	7	12
OmpA domain protein transmembrane	C9QZB9	18	8	2
50S ribosomal protein L10	C9QV92	4.5	2	2
Thioredoxin	C9QX67	3.5	2	1.75
DNA-binding protein HU-alpha	C9QV76	3.5	2	1.75
Glutamate--cysteine ligase	C9R0V6	1	1	1
LPP repeat-containing protein	C9QU30	5	6	0.83
50S ribosomal protein L7/L12	C9QV91	1.5	2	0.75
Outer membrane porin protein C	C9QR92	1	4	0.25

PSM: peptide spectrum matching; super: supernatant; ratio: PSM (pellet)/ PSM (super)

3.3.3 Resolubilization of intact *E. coli* membrane proteins

Protein precipitation with acetone and CMW is deemed to be effective, demonstrating near quantitative yields. However, prior to MS analysis, these precipitated proteins must be resolubilized. In the MS analysis method described above, proteins from the pellet were resolubilized in SDS, and subject to *in-gel* digestion analysis following SDS PAGE. However, it would be desirable to solubilize proteins in an MS-compatible solvent system, so as to avoid the need for SDS-PAGE.

E. coli membrane proteins, as well as yeast whole cell extract (see Section 2.2.2), were chosen as representative proteomes. Precipitation with acetone was selected due to its simplicity and efficiency, together with the fact that more proteins were identified by LC/MS (Figure 3.5). Figure 3.7 demonstrates the re-suspension of *E. coli* membrane or

yeast proteins with increasing amounts of formic acid. Maximum recovery of yeast proteins was obtained at or above 70% formic acid, while for *E. coli* membrane proteome, a more concentrated formic acid solution (80%) is needed to achieve complete re-solubilization. Washburn *et al.* used 90% formic acid for re-solubilization of an integral membrane protein mixture. [62] Although these authors utilized higher concentrations of formic acid than reported in this study, the amount of formic acid needed to completely re-solubilize proteins may vary according to the sample nature.

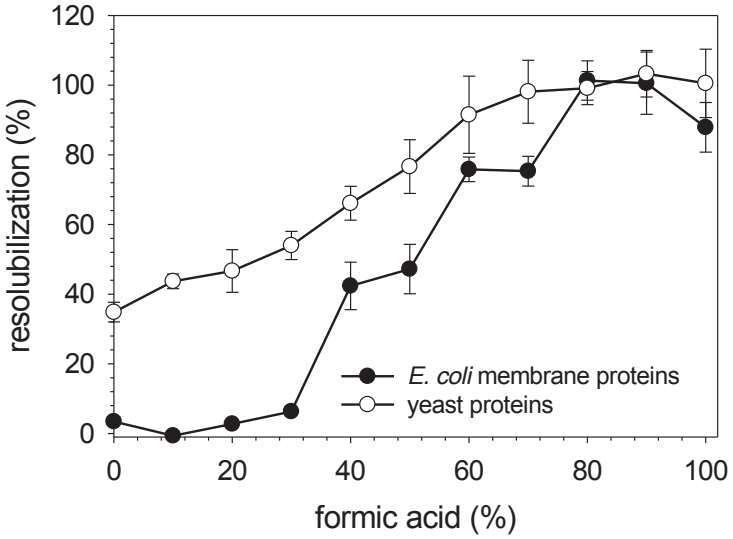

Figure 3.7 Re-suspension of *E. coli* membrane proteins with increasing amounts of formic acid after acetone precipitation. 10 µg/ 100 µL (in 1% SDS) of *E. coli* membrane proteins were precipitated with acetone due to its simplicity and greater ability to precipitate more proteins than CMW (see Figure 3.5). Errors bars, which are representative of the standard deviation of the mean associated with each replicate analysis (5 replicates per sample), are also illustrated.

Traditionally, membrane proteomic analyses on a large-scale may involve the use of organic acids for solubilization. [62] Organic acids (*e.g.* formic acid and acetic acid) are considered compatible with MS and HPLC analysis. However, given the strong acid environment, one might suspect that the use of high concentrations of formic acid may interfere with the elution of proteins in the chromatographic separation. Figure 3.8 shows

the SDS-PAGE of *E. coli* proteins after acetone precipitation and RPLC separation. The control is the *E. coli* proteome mixture before precipitation. The re-suspension of the *E. coli* proteins pellet after acetone precipitation was performed in 80% formic acid and then injected onto RPLC (with a C_{18} column, see Experimental). By similarity with acetone pellet, this gel image indicates that the isolation of *E. coli* proteins through RPLC separation is not harmed by the presence of high concentration of formic acid. Thus, protein samples in the presence of 80% formic acid seem to be compatible with LC/UV.

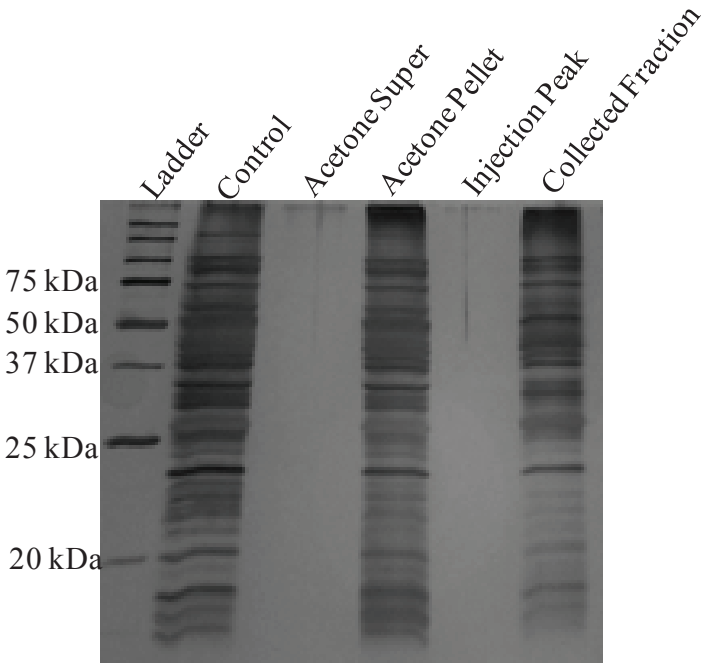

Figure 3.8 SDS-PAGE image of fractions collected following acetone precipitation or RPLC separation. *E. coli* total proteins were used as a representative proteome sample. 10 µg/ 100 µL of proteins were precipitated overnight with cold acetone, and re-suspension of acetone pellet and supernatant was performed with Laemmli buffer. [110] For RPLC separation, samples precipitated with acetone were re-suspended in 80% formic acid and injected directly onto R_2 column. Injection peak and collected peak correspond to isolated fractions from RPLC.

In previous studies, MS-compatible strategies to re-solubilize intact proteins have been applied in the context of top-down proteome analysis. These solvent systems include 8 M urea, [122] as well as 5% acetonitrile/0.1% formic acid. [106] Figure 3.9 provides a

quantitative LC/UV assessment of the intact membrane proteins re-solubilized with 80% formic acid as well as 8 M urea and 5% acetonitrile/0.1% formic acid. Complete membrane protein re-solubilization was achieved with 80% formic acid, while 8 M urea and 5% acetonitrile/0.1% formic acid maintains around 56 ± 3% and 24 ± 0.3% of *E. coli* membrane proteins in solution, respectively. Tran *et al.* developed a 4D separation system toward analysis of intact proteins (top-down experiment) by using 5% acetonitrile/0.2% formic acid for re-solubilization of these proteins. [106] Here, we demonstrate that high concentrations of acid are more efficient than 5% acetonitrile/0.1% formic acid (or 8 M urea) to maintain membrane proteins in solution. The potential of re-solubilization of membrane proteins by 80% formic acid, together with its compatibility with LC analysis may represent a promising alternative for the analysis of intact proteins through top-down MS.

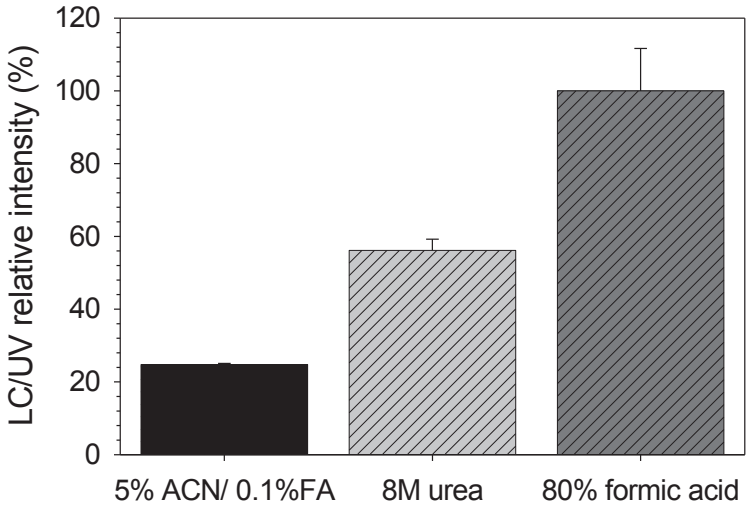

Figure 3.9 LC/UV signal intensity of *E. coli* membrane proteins re-solubilized with different solvents following acetone precipitation. Errors bars, which are representative of the standard deviation of the mean associated with each replicate analysis, are also illustrated here. For each sample, a total of 5 replications were performed. FA represents formic acid and is set at 100%.

As a direct application of the precipitation and protein re-solubilization method, a standard protein, myoglobin (0.5 g/ L), was solubilized in 80% formic acid (before and after precipitation) and subjected to ESI-MS analysis. Fig 3.10 shows the multiple-charge electrospray mass spectrum of myoglobin (in 80% formic acid) obtained following ESI-MS direct injection. The deconvolution of multiply charged states of myoglobin (before [Fig 3.10A] and after acetone precipitation [Fig 3.10B]) can be easily observed in the mass spectrum. It is noted that the signal intensity of the myoglobin sample prepared in formic acid was lower than that of the control. Reduced signal intensity may be related to amount of the residual contaminants (*e.g.* acetone, SDS) and/or denaturation by SDS. Although more studies are needed to confirm this hypothesis, the incorporation of 80% formic acid in the top-down proteome workflow can be recommended as a suitable alternative to re-solubilizing LC/MS-compatible additives.

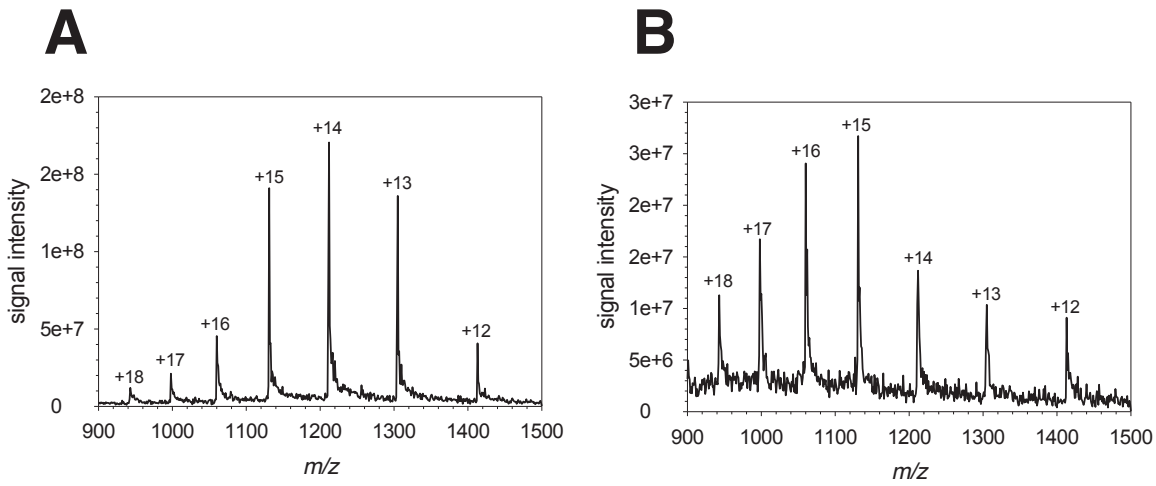

Figure 3.10 Illustration of the multiply-charged electrospray ESI mass spectrum of myoglobin (0.5 g/L) with 80% formic acid before (A) and after (B) acetone precipitation.

3.4 Conclusions

It is a challenging problem to obtain ESI mass spectra of membrane proteins, mainly due to the presence of detergents used to solubilize these hydrophobic proteins. Protein precipitation methods have been described to remove detergent and other interferences prior to mass spectrometry analysis. The challenge is to recovery the initial membrane protein amount after protein precipitation with organic solvents such as acetone and CMW. Here, we reported that high recovery of membrane proteins can be obtained with acetone and CMW methods. This high recovery was only observed after quantification of precipitated membrane proteins by mass spectrometry. Furthermore, LC/UV and MS analysis of intact proteins were acquired following re-solubilization with 80% formic acid.

Chapter 4

Conclusions

4.1 Summary

This report describes work toward the development of a high-throughput proteomics workflow for detection of intact membrane proteins through MS analysis. In Chapter 1, the goals and difficulties of current MS-based membrane proteome experiments were discussed. It presented and highlighted the benefits and disadvantages of studying membrane proteins in large scale. One of the challenges for studying the membrane proteome is to find a method compatible to LC/MS downstream. Currently, the anionic detergent SDS is the widely solvent utilized to keep all membrane proteins in solution. However, this detergent is not compatible with LC or even MS analysis, and thus techniques such as protein precipitation with organic solvents (*e.g.* acetone and CMW) are often applied to sufficiently eliminate SDS prior to LC/MS experiments.

Chapter 2 presented the validation of a MS-compatible surfactants (SDS alternatives), PFOA and APFO, for proteome experiments. Between these fluorinated surfactants, only APFO (instead its acid version, PFOA) was considered favourable for proteome solubilization as efficient as SDS. Unfortunately, APFO was only slightly (twice more than SDS though) compatible with LC/MS analysis. The great advantage of using APFO is that it can be easily removed by a one-step evaporation protocol.

In Chapter 3, the evaluation of current SDS removal protocols as well as alternatives to re-solubilize membrane proteins were presented. In contrast to previous work, it was found that efficient protein recovery was achieved with both acetone and CMW precipitations, even though protein quantification methods (SDS-PAGE, BCA, and

western blotting) showed different values. For resolubilization of intact membrane proteins, 80% formic acid was found to be the most effective solvent (~100% resolubilization). This resolubilization method may be a suitable alternative for intact protein analysis.

4.2 Future Work

The main purpose of this thesis was to develop high-throughput sample preparation methods to analyze intact membrane proteins by mass spectrometry. The analysis of membrane proteins by mass spectrometry is perhaps one of the greatest challenges in the proteomics field, mainly due to their hydrophobic nature which makes them difficult to isolate. While the work presented here focused on an evaluation of alternatives to SDS to solubilize and/or re-solubilize membrane proteins, the use of protein pre-fractionation techniques prior to MS analysis is another issue for analysis of intact membrane proteins (top-down proteomics). The GELFrEE (gel eluted liquid fractionation entrapment electrophoresis) platform [34] has renewed interest in the analysis of intact proteins since the platform recovers intact proteins in solution. Unfortunately, proteins are collected in a solution containing around 0.1% SDS, which interferes with subsequent LC or MS analysis. Thus, an alternative to perform GELFrEE without SDS in the system seems an attractive method.

In Chapter 2, the evaluation of APFO for proteome experiments was executed. In this chapter I verified that this ammonium salt version of PFOA is as effective as SDS to solubilize proteins. As APFO has been used for electrophoresis (perfluorooctanoic acid, PFO/PAGE [84]), we predict that this fluorinated surfactant can replace SDS in the GELFrEE system. The use of this strategy (APFO-GELFrEE) would provide an easier

pathway than the regular GELFrEE system, since the removal of APFO is simpler than SDS. Thus, an evaluation of this APFO-GELFrEE against regular GELFrEE will be performed. Beyond optimization of the system, the *E. coli* membrane proteome will be prefractionated by APFO-GELFrEE and regular GELFrEE and then subjected to mass spectrometry. A comparison of both methods will be evaluated according to number, size and type of proteins identified.

Following the GELFrEE separation of proteins, a common practice is to remove interference (surfactants) prior to MS analysis. For instance, in a regular GELFrEE system the removal of SDS is sometime a difficult task since this anionic detergent firmly binds to cationic amino acids. Also, the method of re-solubilization of proteins plays a crucial role in the protein recovery following protein precipitation with organic solvents. Thus, a common practice in proteome experiments is to re-solubilize proteins in MS-compatible surfactants. As an alternative to SDS, several MS-compatible surfactants have been developed (*e.g.* RapiGest from Waters). These acid labile surfactants are designed to structurally resemble SDS, and consist of an ionic moiety (sulfonate) and a hydrophobic alkyl chain. [82] Beyond the MS compatibility, another advantage it is that trypsin enzyme activity is not significantly affected in presence of 0.1% to 1% RapiGest. [73] With these benefits and the recent proven success of RapiGest to re-solubilize membrane proteins, [72] this MS-compatible surfactant as well as 8 M urea and 80% formic acid will be used to maintain intact membrane proteins in solution following detergent removal. Figure 3.10 provides an intact protein analysis through MS direct infusion in the presence of 80% formic acid before and after acetone precipitation. For more reliable results, top-down experiments are often executed through the LC/MS platform. The LC separation prior to

MS reduces the complexity of the sample and avoids possible interfering compounds. Thus, a top-down proteomics workflow for membrane proteins will be evaluated by using GELFrEE- LC/MS platform. This top-down workflow will be used to complement Section 3.3.3.

While work remains to tackle issues of sample preparation for membrane proteome analysis, it is anticipated that the analysis of the membrane proteome will be facilitated through the technologies developed here. Thus, it is hoped that this research will establish a proteomic workflow in order to study membrane proteins, and therefore help biomarker discovery studies.

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