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Review

Advances in shotgun proteomics and the analysis of membrane proteomes

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ABSTRACT

The emergence of shotgun proteomics has facilitated the numerous biological discoveries made by proteomic studies. However, comprehensive proteomic analysis remains challenging and shotgun proteomics is a continually changing field. This review details the recent developments in shotgun proteomics and describes emerging technologies that will influence shotgun proteomics going forward. In addition, proteomic studies of integral membrane proteins remain challenging due to the hydrophobic nature in integral membrane proteins and their general low abundance levels. However, there have been many strategies developed for enriching, isolating and separating membrane proteins for proteomic analysis that have moved this field forward. In summary, while shotgun proteomics is a widely used and mature technology, the continued pace of improvements in mass spectrometry and proteomic technology and methods indicate that future studies will have an even greater impact on biological discovery.

Contents

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1. Introduction

After the advances and achievements of genomics, the study of proteins encoded by the genome was the next logical step in understanding functional aspects of the cell. The study of all proteins encoded by the genome, called proteomics, is a daunting task in and of itself. Unlike the genome, the proteome is very dynamic and can change in response to cellular or environmental factors. In addition, the proteome is orders of magnitude more complex than the genome due to processes such as splicing, post-translational events at the protein level, post-translational modifications, protein degradation, drug perturbations and disease. Furthermore, differences in protein abundance within the cell vary greatly and the challenge of identifying low abundant proteins remains a challenge.

Advances in mass spectrometry (MS) have been crucial for the proteomic field. It is the driving force behind the ability to identify low abundant proteins in such complex mixtures. There are several MS technologies used, as well as combining MS with other analytical protein technologies. Due to the complexity of proteomic samples, the ability to separate proteins and peptides prior to analysis by mass spectrometry is critical. There are a wide variety of separation techniques that have been utilized in proteomic analyses over the years. These include two-dimensional gel electrophoresis (2D-PAGE) for protein separation (reviewed in [1]) and multidimensional liquid chromatography separation techniques (reviewed in [2]).

In the 1970s, protein mixtures were often analyzed using 2D-PAGE [3–5], which allowed for the separation of a large number of proteins as well as representing their abundance. This approach involves separating proteins by their isoelectric point (p*I*) in the first dimension, followed by SDS-PAGE to separate them according to their molecular weight in the second dimension. In the 1980s, the idea of building protein databases was proposed using subtractive pattern analysis of these gels [6–8]. Over time, with the advances in analytical protein technologies and advancements in mass spectrometry, it became possible for many proteins to be resolved by 2D-PAGE. 2D-PAGE remains an important tool in the proteomic arsenal (for a review see [1]). However, the disadvantages of 2D-PAGE include a large amount of sample handling, a limited dynamic range, and difficulties resolving low abundance [9–11] proteins with extreme p*I* and molecular weights [12,13], and hydrophobic proteins such as membrane proteins [14]. These issues led to development of alternative solutions for proteome analysis resulting in the development of shotgun proteomics.

Shotgun proteomics is analogous to shotgun sequencing where DNA is broken into small fragments; these fragments are sequenced, and recombined in silico to determine the DNA sequence of an organism. In a generalized shotgun proteomic pipeline, a mixture of proteins is digested into peptides (using proteases such as trypsin), the peptides are loaded onto at least a two-dimensional chromatography based separation system, peptides are eluted into a tandem mass spectrometer in an automated fashion, and the resulting tandem mass spectrometry data is analyzed by powerful computational systems. Large scale shotgun proteomics effectively began with the introduction of multidimensional protein identification technology (MudPIT) [15–17]. In MudPIT, a microcapillary column is packed with reversed phase (RP) and strong cation exchange (SCX) packing material, loaded with a complex peptide mixture and placed in line between an HPLC and a tandem mass spectrometry system (for review see [2]). Two areas where MudPIT resulted in significant improvements over 2D-PAGE approaches, for example, were in the detection and identification of membrane proteins and low abundance proteins [16]. The success of the MudPIT approach for the large scale analysis of the yeast [16], malaria [18], and rice [19] proteomes, for example, has led to the development of many coupled two-dimensional separation and mass spectrometry systems (reviewed in [2]). This review will provide a selected update on shotgun proteomics and the direction that the field is moving. Also, since the analysis of membrane proteins remains a significant challenge in proteomics, this review will discuss recent developments and applications of shotgun proteomics to the analysis of membrane proteins.

2. Recent improvements in shotgun proteomics

In spite of the development of shotgun proteomic approaches, comprehensive proteome coverage remains a challenge. As a result there is continual research into improved methods for separation of peptides, mass spectrometry systems and data analysis tools. All of these areas are active areas of research and cannot be comprehensively covered in a single review. Given the challenges of complete proteome coverage and the extraordinary efforts that go into comprehensive proteome coverage [20], researchers are continually pursuing improvements in shotgun proteomic techniques. Clearly, one area of continued research is in the proteome informatics needed to analyze proteomic datasets. However, this is beyond the scope of this review but is covered in reviews in this issue [21–23]. Here the discussion will be limited to selected improvements in mass spectrometry and chromatography systems and the move from qualitative to quantitative shotgun proteomics.

2.1. *Expansion of shotgun proteomic systems*

Initially, large scale shotgun proteomics was defined as an SCX/RP/MS/MS system which includes MudPIT and other several column configurations coupling SCX and RP chromatography (reviewed in [2]). Alternative configurations to SCX with RP were also investigated that included the use of anion exchange chromatography and RP, affinity chromatography (AC) and RP, isoelectric focusing (IEF) and RP, capillary

electrophoresis (CE) and RP (reviewed in [2]). Recently, additional approaches have been added to the repertoire of shotgun proteomic approaches that are being increasingly adopted (Table 1).

To begin, one approach is now commonly referred to as GeLC–MS/MS [24–26]. In GeLC–MS/MS, proteins are initially separated by size on an SDS-PAGE gel. The gel is then sliced at specific intervals resulting in the entire gel being analyzed rather than specific bands being analyzed [24]. The proteins in each gel slice are digested and analyzed by RP–MS/MS. This is different from most shotgun proteomic approaches where peptides are separated in two dimensions. Nevertheless, the GeLC–MS/MS approach is now generally considered a shotgun proteomic approach.

To develop another alternative shotgun proteomic approach, Gilar and colleagues evaluated several two-dimensional chromatography separation systems in a theoretical and experimental publication [27]. In this body of work they found that a two-dimensional chromatography RP–RP system, each containing a different pH, compared favorably to SCX–RP systems [27]. Further development and evaluation of this approach led to the proposal of an RP–RP/MS/MS system for proteomics where the first RP column uses a pH of 10 and the second RP column uses a pH of 2.6 [28].

The fact that large scale shotgun proteomic approaches include many approaches like SCX–RP/MS/MS [16], RP–RP/MS/MS [28], GeLC–MS/MS [24], and several others reviewed in [2] (Table 1) begets the question which approach should one use. A recent area of study has therefore been to compare and contrast different shotgun proteomic approaches and potentially determine which system is optimal for proteome analysis [29–37]. However, the outcome of these studies depends on the objectives of the research groups. For example, is one trying to optimize sensitivity or reproducibility or both? In one study, peptide SCX and peptide IEF separations prior to LC–MS/MS were compared with the objective of determining which approach was the most reproducible for biomarker discovery [36]. Here the authors found peptide IEF to outperform SCX [36]. In another study to determine which platform had the highest number of protein identifications, a commercial OFFGEL device was compared to MudPIT with both systems having comparable results in terms for protein identifications, peptide identifications, and reproducibility [31]. The RP–RP/MS/MS system [27,28] has been shown to outperform SCX–RP approaches in some studies [29,34]. Finally, the GeLC–MS/MS approach, where proteins are first separated by SDS-PAGE followed by peptide analysis by RP–MS/MS, has also outperformed SCX [32,35] and IEF [32]. While SCX has not performed as well as several approaches in these studies, it is important to keep in mind that there are several different SCX based approaches with different performances that include MudPIT, off line SCX, and on line SCX with an RP trap (reviewed in [2]). Depending on which SCX approach was used will influence the comparative analyses. That being said, there are clearly several powerful approaches for the analysis of proteomes that include SCX/RP/MS/MS, IEF–RP/MS/MS, RP–RP/MS/MS, and GeLC–MS/MS. Depending on the existing expertise of a given research group that wishes to carry out more proteomic research, one could adopt one of these pipelines that best matches their prior expertise.

2.2. *Developments in mass spectrometry and chromatography that impact shotgun proteomics*

There are routinely new developments in mass spectrometry and HPLC systems for use in proteomics. Often, these systems are developed by instrumentation vendors first and then take time to become widely adopted. For example, the LTQ-Orbitrap system for high mass accuracy, without the need for use of liquid nitrogen or liquid helium [38], has been widely adopted. The potential advantages of this system and high mass accuracy in general, have been recently reviewed [39,40]. The coupling of this system to shotgun proteomic approaches promises to provide larger scale datasets than previously achieved [20]. An additional improvement that is being investigated is the implementation of high field asymmetric waveform ion mobility which has been shown to increase both the peak capacity and dynamic range of shotgun proteomic analyses [41]. In addition to mass spectrometry system developments, improvements to HPLC systems also occur. One potentially valuable tool is the development and implementation of ultra high-pressure systems that could yield more protein identifications than the use of standard HPLC systems [42]. However, these systems are expensive, with some still in early development, and it will be several years before the potential is realized of UPLC coupled with advanced mass spectrometry systems.

Table 1 – 2D separation techniques for shotgun proteomics.

SCX/RP/MS/MS	Strong cation exchange/reverse phase chromatography is coupled together as seen in MudPIT. Peptides are separated using 2D chromatography directly coupled to the mass spectrometer for MS/MS analysis.	Reviewed in [2]
IEF–RP/MS/MS	Isoelectric focusing is performed offline. Isolated fractions are digested and subjected to RP chromatography followed by MS/MS.	[31,130]
RP–RP/MS/MS	Coupled reverse phase chromatography, with the first RP being offline has also been optimized for shotgun proteomics. Using a different pH for each RP separation was found to be an improvement for this approach.	[28]
GeLC–RP/MS/MS	GeLC involves separating proteins by SDS-PAGE. Proteins are excised from the gel and digested. Peptides are then separated by RP chromatography followed by MS/MS.	[24–26]
AC–RP/MSMS	Affinity chromatography has been used to study post-translational modifications. Specific affinity resin is used to enrich phosphopeptides and glycopeptides for shotgun proteomic analysis. Isolated peptides are further separated using RP chromatography.	Reviewed in [2]
CE–RP/MS/MS	Capillary electrophoresis has also been coupled to RP chromatography. These two separation techniques are coupled together in line with the mass spectrometer.	Reviewed in [2]
AE–RP/MS/MS	Anion exchange chromatography has been used offline for the separation of peptides. Peptides can then be eluted and fractions subjected to RP chromatography and MS/MS analysis.	Reviewed in [2]

Optimization of the use of mass spectrometers is also one mechanism by which improved shotgun proteomic analyses can be achieved. An example of this is gas phase fractionation (GPF) where peptides are analyzed in successive runs only in certain mass to charge range windows [43]. Optimized use of GPF can be achieved by analyzing the genomic complexity of an organism and experimental data generated from test runs [44]. The potential impact of GPF in a shotgun proteomic pipeline was prominently demonstrated in a comprehensive quantitative analysis of haploid versus diploid yeast [20]. In ion trap mass spectrometers, dynamic exclusion is used to prevent the repetitive detection of the most abundant proteins in a shotgun proteomic analysis. However, recently the effect of dynamic exclusion times on shotgun proteomic results has been considered, and an approach proposed to maximize the dynamic exclusion time for the optimal detection and identification of peptides [45]. These are two examples of recently improved methods that can be used on mass spectrometry systems already in use that can improve shotgun proteomic analyses.

An additional improvement is the development of novel peptide fragmentation techniques. Electron transfer dissociation (ETD) is one such development [46,47]. Initially the focus for the use of ETD was on the facilitated analysis of phosphopeptides [48]. However, the complementary of ETD to the more traditional collision activated dissociation (CAD) has led to the development of decision tree driven tandem mass spectrometry [49]. Here, a mass spectrometer determines, on the fly, the nature of a peptide charge state and mass to charge ratio and then uses either CAD or ETD to generate the optimal peptide fragmentation [49]. The implementation of this approach led to the analysis of 2496 yeast proteins and 3329 human embryonic stem cell proteins [49]. An additional use of the decision tree approach and multiple enzyme digestion led to the detection and identification of 3313 yeast proteins [50]. The adoption of this approach by other researchers could add significantly to the proteins detected and identified in a shotgun proteomic analysis.

2.3. *Quantitative shotgun proteomics*

Large scale shotgun proteomics began with the qualitative description of proteomes where lists of detected and identified proteins were generated (for many examples see [2]). However, one can argue that proteomics in general is moving towards always being quantitative. The majority of studies in current proteomics are quantitative rather than qualitative, and this trend will likely continue and may reach the point where qualitative proteomics is rarely under taken. There are two common ways of quantifying proteomic data, one is using isotopic labeling and the other is label free (reviewed in [51]). Labeling with isotopes requires that samples are labeled with different isotopes and then combined before sample preparation and MS analysis (reviewed in [51]). Label free quantitation is based on spectral counting, peak area or peak intensity (reviewed in [51]). Values are then directly compared between samples. Quantization by spectral counting is achieved by comparing the number of MS/MS spectra for a given protein between samples [52]. A wide variety of quantitative proteomic methods have been developed over the years (reviewed in [51]), and this is beyond the scope of this review. However, these methods have been widely used in shotgun proteomic approaches.

Shotgun proteomic approaches are commonly used in quantitative proteomic studies. MudPIT has been used to analyze membrane proteome changes [53], changes in transcriptional regulatory complexes [54], a time course analysis of rapamycin treatment [55], plant pathogen life cycle stages [56], and changes in the proteomes of isotopically labeled animals [57,58]. Non MudPIT SCX/RP/MS/MS approaches were recently used to analyze mesenchymal stem cell differentiation [59], prostate cancer cells [60], and the mTOR controlled nuclear changes in HeLa cells [61]. GeLC/MS/MS approaches have been used to analyze MCF-7 cells [62], nucleolus changes in adenovirus infected cells [63], E3 ubiquitin ligase substrates [64], and DNA damage induced protein subcellular localization changes in human colon carcinoma cells [65]. These are but a few of the many examples where shotgun proteomic approaches have been used in valuable quantitative proteomic studies.

3. Membrane proteins and membrane protein proteomics

3.1. *Challenge facing membrane proteins*

Integral membrane proteins (IMPs) are among the most important proteins within a cell due to their strong implication on cell survival. They represent one-third of the proteins encoded by the human genome, and are many times underrepresented in proteomic profiles [14,66–68]. IMPs encompass a variety of functions required by the cell, including: 1) communicating with the cells external environment, 2) dictating immune response recognition, 3) targeting for most pharmaceutical drugs, 4) controlling cell adhesion to form tissues, and 5) controlling metabolic processes (i.e. salt balance).

IMPs can easily be characterized by their structural features. They are amphipathic (containing regions that are hydrophobic and hydrophilic) and have one or more polypeptide chains passing through the membrane, either as α-helices or β-strands. It is the amphipathic nature that contributes to the difficulty of studying membrane proteins. The hydropathy pattern of β-barrel IMPs tends to be very similar to soluble proteins and does not present an analytical challenge [69]. However, α-helical IMPs pose difficult challenges to the proteomic characterization of these proteins. Moreover, membrane proteins are low abundant in nature, which also attributes to the challenge of a proteomic characterization.

3.2. *Enrichment of membrane proteins*

Because of the low abundant nature of IMPs, enrichment of these proteins is crucial for proteomic analysis. There are several common strategies for enriching IMPs and are often used in combination with one another (Table 2): subcellular fractionation [70–72], removal of membrane-associated proteins [69,73,74], proteinase K "shaving" [75], delipidation [76,77] and affinity purification [66,69,74,78–84] (Fig. 1). One simple way of isolating membrane proteins is removing the cytosolic and membrane-associated proteins. This can

Table 2 – Enrichment and separation techniques for membrane shotgun proteomics.

Membrane protein enrichment		
Subcellular fractionation	Achieved by separating out subcellular compartments with a series of centrifugations at increasing speeds.	[70–72,153]
Delipidation	The removal of the lipid bilayer surrounding the transmembrane helices or strands.	[76,77]
Affinity purification	Typical affinity purifications of transmembrane proteins involve the use of biotinylation and glycosylation affinity purifications. Biotinylation targets primary amine containing molecules while glycosylation affinity purifications target glycosylated membrane proteins.	[66,74,78–84]
Removal of non-membrane proteins	The use of high salt and high pH has been successful in removing cytosolic and membrane-associated proteins.	[69,73,74]
Membrane protein separation (gel-based)		
Blue-native electrophoresis	BNE has been proven successful in separating out membrane proteins. The use of CBB is important for applying an overall net negative charge to allow proteins to be separated by migration to the anode rather by their intrinsic p*I*.	[107–110,125]
High resolution clear native	hrCNE is very similar to BNE except that CBB is not need. hrCNE uses non-colored mixed micelles to induce a net negative charge of the proteins.	[111]
GeLC–MS/MS	SDS-PAGE has been used to separate membrane proteins by size. Sections of the gel are removed and in-gel digestions using proteases are performed prior to mass spectrometry analysis.	[24–26,106]
Membrane protein separation (solution-based)		
Multidimensional protein technology	MudPIT is a 2D chromatographic approach to separating proteins in shotgun proteomics. Proteins are first digested into peptides and they are separated using strong cation exchange and reverse phase chromatography.	[16,17]
Elevated temperature	Elevating the temperature of your sample during 1D or 2D separations can increase peak selectivity and peak resolution. This is beneficial for identifying more membrane proteins in shotgun proteomic studies.	[76]
Immobilized pH gradient isoelectric focusing	Digested membrane proteins can be separated by IPG-IEF in the presence of 60% methanol with relatively good success.	[130]
OFFGEL fractionation	OFFGEL fractionation was developed by Agilent Technologies which involves separating out proteins by IPG-IEF. This approach gives similar results to MudPIT for the analysis of membrane proteins.	[31]

obviously be used in conjunction with other membrane enrichment strategies to help clean up isolated membrane fractions. Because of the hydrophobic nature of IMPs, solutions containing specific detergents or organic solvents used for stabilization and solubility and high salt solutions are typically not helpful. However, high salt conditions can help solubilize and disrupt electrostatic protein–protein interactions. Methods reported in the literature use sodium carbonate or sodium hydroxide at high pH to successfully remove non-membrane proteins [69,73]. Others have used ice-cold 1 M KCL and 0.1 M Na_2CO_3 at high pH [74].

During subcellular fractionation, cells are lysed and subcellular components are separated by a series of centrifugations at increasing speeds. Isolating membrane proteins in single-cell organisms requires only lysis of the cells followed by sequential centrifugation steps to separate the soluble and insoluble proteins. In multicellular organisms, there is another level of separation that is necessary for isolating tissues, organelles and proteins. The most widely used method for fractionating plasma membranes and organelles is density gradient centrifugation (using sucrose or sorbitol) (Fig. 1A). This method has been shown to enrich plasma membranes; however, they can be contaminated with organellar membranes such as mitochondrial, Golgi and ER membranes [70,71]. It is difficult to obtain well separated IMPs from organelles because of the close proximity and associations between them, such as the nuclear membrane and the ER [66]. In addition, separating out highly abundant non-membrane proteins, such as cytoskeletal proteins, also remains a challenge [85,86]. Two other methods that have been successful in fractionating the plasma membrane are the cationic colloidal silica technique and aqueous polymer two-phase system.

The cationic colloidal silica technique consists of coating cells with a dense pellicle of silica and poly anions [87,88]. This technique is based upon the ionic interaction of the plasma membrane with positively-charged silica. The binding of pellicle enhances the density of the plasma membrane and stabilizes it against vesiculation and lateral reorientation. Once the cells are lysed, the plasma membrane can be isolated by centrifugation. This has been shown successful for isolating plasma membrane proteins [89,90] and tissue samples [91,92] for mass spectrometry analysis.

The aqueous polymer two-phase system separates plasma membranes based on the surface properties of IMPs [93]. This method requires the mixture of two water-soluble polymers so that distinct phases are formed [93]. For the fractionation of plasma membranes, the most commonly used polymers are polyethylene glycol (PEG) and dextran. Membranes will fractionate into the PEG (hydrophobic phase), while soluble proteins will partition into the dextran phase. Using the two-phase system in combination with solubilization procedures has been efficient for enriching membrane proteins for proteomic studies [94].

Isolating IMPs can also be accomplished by removing the lipid bilayer that surrounds the transmembrane α-helices or

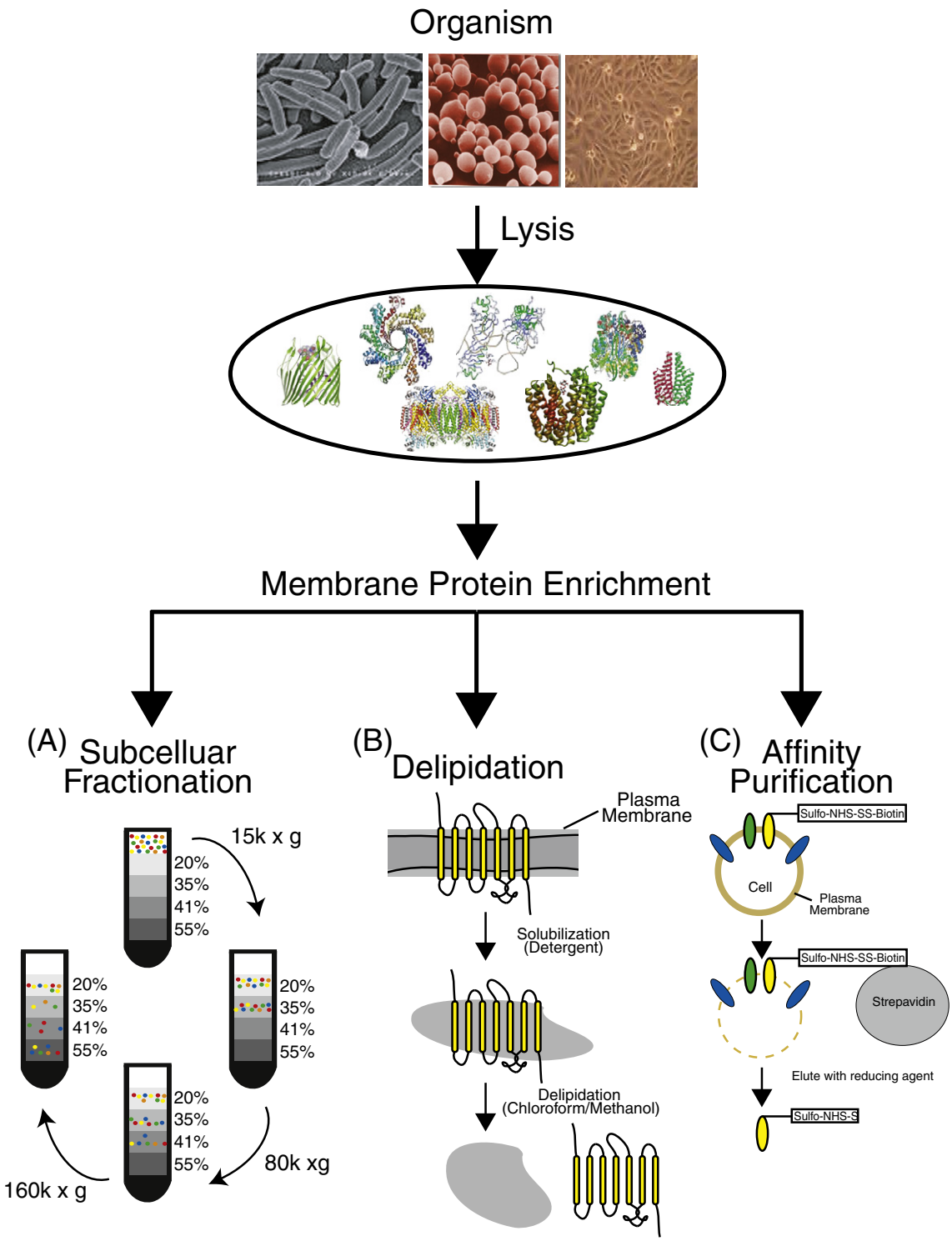

Fig. 1 – Membrane protein enrichment. It is vital to perform a membrane enrichment step for proteomic analysis of membrane proteins. A) Subcellular compartments are separated by increasing speeds using a glycerol or sorbitol gradient. After each speed, organelles are sedimented to the bottom of the tube and recovered in the pellet. This allows for the enrichment of different organellar membrane subtypes [70–72,153]. B) The process of delipidation allows for the separation of the proteins from a lipid bilayer. Solubilization of the membrane in the presence of detergent is performed followed by delipidation using a chloroform/methanol solution to extract and solubilize membrane proteins from the lipid bilayers [76,77]. C) Affinity purification of membrane proteins using biotinylation. The membrane protein is tagged with Sulfo-NHS-SS-Biotin, which contains a thiol-cleavage group. The biotin tagged membrane protein is enriched using streptavidin resin, washed to remove proteins that are non-specifically binding and eluted using a reducing agent such as dithiothreitol (DTT) [66,74,81–84].

β-strands (Fig. 1B). This can be done in combination with subcellular fractionation and allows easier solubilization of the IMPs [76,77]. Lipids can be extracted using a solution containing chloroform and methanol to precipitate out the IMPs [95]. The lipids are extracted into the chloroform fraction while proteins are precipitated at the chloroform/methanol interface [95]. This technique allows the solubilization of IMPs to be reconstituted using MS compatible buffers such as ammonium bicarbonate buffers [77].

Integral membrane proteins can also be isolated using purification methods. Labeling cell surface proteins has emerged as an important tool in isolating and studying IMPs. Several methods have been employed including biotinylation affinity purification [66,69,74,81–84] (Fig. 1C), glycosylation affinity purification and chemical capturing [78–80,96,97]. The extracellular portions of IMPs are often modified by PTMs. N-glycosylation is a prominent modification that plays a significant role in the assembly of multicellular organs and organisms [98]. Over the past decade, liquid chromatography coupled with mass spectrometry (LC–MS) has played an important role in determining *in vivo* glycosylation sites [99–101]. Enrichment of glycoproteins can be done through two major approaches: lectin mediated capture and cell surface-capturing (CSC). [78–80,96,97,102]. Recently, a new method called filter aided sample preparation (FASP) was developed for mass spectrometry-based proteomics [103]. FASP uses a common filtration device for solubilization, buffer exchange, chemical modification and protease digestion of proteins [103]. Zielinska et al. combined FASP with lectin mediated capture for the isolation of glycopeptides [102]. In this procedure, lectin was added to the top of the filter after on-filter protein digestion [103]. The glycosylated peptides were bound to lectin and were retained on top of the filter, whereas the unbound peptides were washed through. The glycopeptides were then deglycosylated by PNGase F, eluted and analyzed by mass spectrometry [103]. The authors indicated that all three classes of N-glycosylated peptides can be isolated using multiple lectins [103].

Biotinylation reagents are targeted to primary amine containing molecules (i.e. lysine residues) on cell surface exposed proteins. One common reagent used for biotinylation is Sulfo-NHS-SS-Biotin (as reviewed in [104]) (Fig. 1C). This is a thiol-cleavable reagent that contains a spacer to relieve steric hindrance. It is water soluble and is charged by a sodium sulfoxide group on the succinimidyl ring that prevents its ability to permeate the cell membrane, creating specificity for exposed proteins on the cell surface (as reviewed in [104]). The biotinylated membrane proteins can then be enriched by affinity purification using avidin or streptavidin resin [66,69,74,81–84]. The addition of reducing agent allows for the elution of the isolated IMPs. One disadvantage of this method is the contamination of biotinylated proteins from non-viable cells which have lost their structural integrity [104]. Moreover, labeling may not be efficient for cell surface proteins which contain few or no lysine residues [104]. Nonetheless, biotinylation has been a proven method for enriching IMPs. More IMP identifications have come from the biotinylation affinity purification, whereas glycosylation affinity purification is more efficient for isolating IMPs containing glycosylated modifications.

3.3. *Gel-based separation approaches for membrane proteins*

The ability to separate membrane proteins for proteomic analysis is challenging. One of the major issues with analyzing membrane proteins by proteomics is the solvent compatibility issue. Because of the hydrophobic nature of IMPs, certain detergents and strong solubilization solutions are necessary to provide stability while in solution. Unfortunately these chemicals can interfere with mass spectrometry analyses. Initially, 2D-PAGE gels were used to separate out IMPs for proteomic analysis (as reviewed in [105]). However, there are difficulties extracting and solubilizing them in the isoelectric focusing (IEF) buffer (as reviewed in [105]). Typical buffers for IEF include nonionic detergents such as urea and CHAPS, neither of which stabilize membrane proteins [14]. Most solutions used to solubilize membrane proteins contain SDS, however, SDS is incompatible with IEF. In addition, membrane proteins aggregate as they approach their p*I* during IEF. Therefore, 2D-PAGE is not an optimal separation technique for membrane proteins. There are however other gel-based separation methods and gel free separation methods that have been successful.

An earlier approach for separating membrane proteins using SDS-PAGE prior to mass spectrometry analysis was developed by Simpson et al. [106]. Compared with 2D-PAGE, this approach solely relies on the size of the proteins for separation. Simpson et al. isolated plasma membrane proteins from a human colon carcinoma cell line and subjected them to SDS-PAGE [106]. The gel was cut into slices that covered known apparent mass ranges. In gel digestion with trypsin was performed and the peptides were extracted from the individual gel slices. The peptides were analyzed using MS/MS and identified with a non-redundant protein database using SEQUEST [106]. This approach, now known as GeLC–MS/MS, has been widely used for the proteomic analysis of membrane proteins.

To improve on gel-based approaches for membrane protein analysis, blue-native electrophoresis (BNE) and clear-native electrophoresis (CNE) are the techniques used to separate out native proteins and protein complexes [107–110]. The analysis of membrane proteins using BNE requires several components: 1) anionic coommassie brilliant blue G-250 (CBB) must be added to the membrane proteins to apply an overall negative charge to the protein surfaces, 2) there needs to be a buffering component to regulate pH between 7.0 and 7.5, such as Bis–Tris, and 3) there needs to be a zwitterionic compound present to improve membrane solubilization [108]. Once membrane proteins have been isolated, CBB is then added to produce an overall negative charge on the protein surfaces [107–109]. This allows less aggregation between proteins due to charge repulsion [107–109]. Therefore, membrane proteins that are bound to CBB behave more similarly to water-soluble proteins [111]. The net negative charge of the proteins allows them to migrate to the anode instead of being separated by their intrinsic p*I* [111]. In addition, CBB can also bind to soluble proteins with basic p*I*s [112]. These also will migrate to the anode increasing the number of contaminants [111]. There are some proteins that cannot be separated by BNE because they cannot bind CBB and, in addition, they have a neutral or basic

pI [111]. These proteins migrate towards the cathode and will be lost in solution. Because BNE was primarily developed to isolate membrane proteins from mitochondria [113], these proteins may be most prominent on the gel. Therefore, whenever non-mitochondrial membrane proteins are of interest, care should be taken to remove the mitochondria using centrifugation [111].

BNE is an efficient tool for isolating protein complexes from biological membranes. However, a complete separation of proteins cannot be expected. To remedy this, BNE can also be used in combination with SDS-PAGE (BNE/SDS-PAGE) as a 2D separation technique [114]. Others have also used BNE/BNE as a 2D separation technique for the analysis of a variety of samples (reviewed in [109,115]. Some disadvantages of BNE include the generation of micelles when combining CBB with neutral detergents [111]). The CBB also interferes with fluorometric and catalytic assays [111].

CNE is very similar to BNE with the exception of CBB not being added to the samples prior to running the gel. Therefore, a net negative charge shift does not occur and separation is solely based upon the pI of the proteins [111]. In addition, CNE can only separate acidic proteins with pIs<7 [111]. Proteins with basic pIs migrate to the cathode and are lost into the running buffer [111]. CNE is also not ideal for separating out membrane proteins. Since most membranes appear as a smear when using this technique [111]. Benefits do include being able to perform fluorometric and catalytic assays because of the lack of CBB present [110].

A new technique called high resolution clear-native electrophoresis (hrCNE) was developed to combine the advantages of both CNE and BNE [112,116]. This approach allows the high resolution separation as BNE but without using CBB [111]. The hrCNE approach uses non-colored mixed micelles to induce a net negative charge of the proteins (reviewed in [111]). Therefore hrCNE will allow the performance of fluorometric and catalytic assays and generate high resolution separation of the proteins similar to BNE [111]. Although hrCNE is an improvement of CNE, it does favor the dissociation of labile proteins from proteins complexes more than BNE [111].

3.4. *Gel-free based separations for membrane proteomics*

Shotgun proteomics is a powerful alternative to gels for looking at integral membrane proteins. Solution based separations such as MudPIT have helped diminish IMP insolubility problems that are typically encountered [16,75]. An early MudPIT analysis of the yeast proteome included a substantial number of membrane proteins [16]. In this analysis, a yeast membrane fraction was solubilized in 90% formic acid in the presence of cyanogen bromide (CNBr), and solubilization using organic acid in the presence of CNBr allowed the membrane embedded proteins to be cleaved [16]. Further proteolytic digestion using endoproteinase LysC and trypsin produces smaller peptides that were then separated using MudPIT and finally analyzed by tandem mass spectrometry [16]. Solution based shotgun proteomic methods allow for the digestion of IMPs, and thereby avoid the necessity of extracting proteins from a gel. Most large scale studies using MudPIT contain the detection and identification of membrane proteins, including analyses of the rice proteome [19], malaria proteome [18], nuclear membranes [117], and the rat brain [118], for example. There have been several shotgun separation methods developed for the analysis of membrane proteins including: 1) organic solvents [119], 2) organic acids [120], 3) detergents [121], and 4) microwave assisted acid hydrolysis [122].

A further advance in the MudPIT analysis of membrane proteins was achieved with the adoption of a high pH carbonate wash method [75]. In this study, membrane proteins were first isolated by homogenizing membrane vesicles using a high pH buffer containing sodium carbonate [75]. This results in membrane vesicles (i.e. lipid bilayers) or membrane sheets that contain integral membrane proteins [75]. Proteinase K is then added to the membrane sheets for digestion of the surface exposed regions of the IMPs [75]. Proteinase K is a non-specific protease which can be problematic for creating large enough peptides for mass spectrometry analysis, however at high pH, proteinase K activity is inhibited so that optimal length peptides are generated. This analysis is only for the surface exposed regions of IMPs [75]. Coupling this approach to MudPIT resulted in the identification of 454 proteins that had predicted transmembrane domains (TM) [75]. However, when using this method care must be taken when choosing buffers to avoid cell lysis and assure membrane stability, or an increase in intracellular protein contamination will occur [123–125]. In addition, due to the presence of PTMs on the surface exposed loops of IMPs, the proteinase K cleavage site may be masked so that cleavage does not occur.

It has long been realized that increasing temperature (typically 30–80 °C) is known to improve peak selectivity and resolution in LC techniques [126,127]. Speers et al. applied this approach to look at membrane proteins using shotgun proteomics [76]. They took an enriched plasma membrane fraction from HeLa cells and performed two separate digestions: a trypsin digest (cleavage after arginine and lysine residues) and a membrane embedded peptide (MEP) digest with proteinase K (non-specific cleavage) [75]. The digested proteins where loaded onto a 100 μM fused silica column containing C18 reversed phase (RP) resin, and a block style column heater was used to control the temperature of the microcapillary column [76]. Protein identifications increased 38% and peptide identifications increased 36% as the temperature was increased from 20 °C to 40 °C [76]. In the MEP analysis, there was a 4-fold increase in protein identifications and a 5-fold increase in peptide identification from 20 °C to 60 °C [76]. The widespread adoption of this approach would likely greatly increase the detection and identification of IMPs.

An alternative approach for separating out peptides or proteins is immobilized pH gradient isoelectric focusing (IPG-IEF) [128,129]. In an application of this approach for the analysis of membrane proteins [130], membrane proteins were isolated from rat livers using a modified sodium carbonate stripping method [131,132]. The membrane proteins were digested using trypsin with different concentrations of methanol (0%, 40% and 60%), and the three digests were used to hydrate linear pH 3–10 IPG strips [130]. Isoelectric focusing was performed for 6 h at room temperature after which the strips were cut and the peptides were extracted [130]. Each

sample was concentrated and desalted for mass spectrometry analysis. The data was filtered using peptide p*I* as an initial filtering criterion followed by imposing a 1% protein FDR cutoff [130]. A total of 1549 unique proteins were identified, including 690 integral membrane proteins [130]. The authors concluded that the addition of 60% methanol allowed an increased number of identifications from 0% to 40% [130].

3.5. *Digestion techniques for membrane protein proteomics*

A great deal of work has been done over the last decade for isolating and separating membrane proteins for proteomic analysis. Only in the past few years has there been an effort to optimize the proteolytic digestion process prior to MS analysis [133–135] (Fig. 2). Trypsin digestion is typically the first step in the proteomic analysis of proteins by mass spectrometry. However, membrane proteins tend to lack trypsin cleavage sites, which results in large peptide fragments that retain their hydrophobic nature and are ultimately not detected by the mass spectrometer.

Recently less specific proteases such as proteinase K, elastase and pepsin have been used for the proteomic analysis of membrane proteins [69,134,135]. As previously mentioned, proteinase K has been used to shave off soluble regions of membrane proteins for MS analysis [75]. Proteinase K is a non-specific protease and is prone to producing a much larger

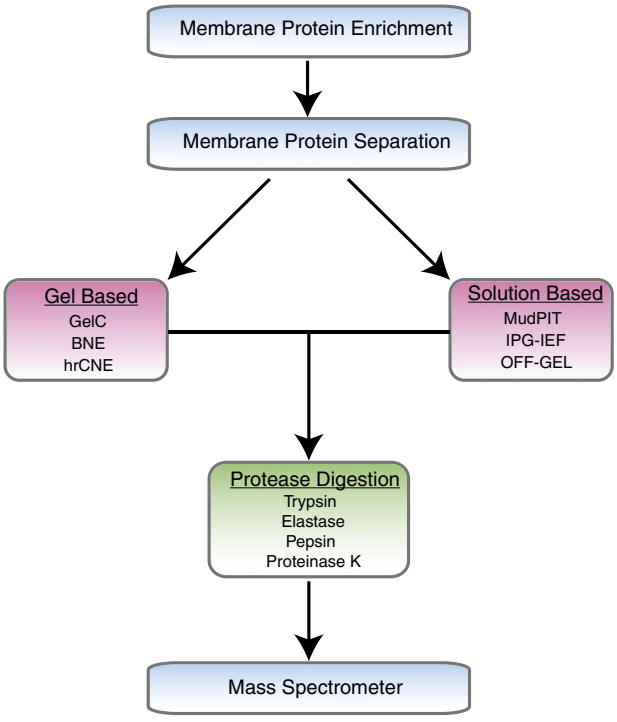

Fig. 2 – A schematic indicating the necessary steps for proteomic analyses of membrane proteins. After the enrichment of membrane proteins, separation methods are used for isolating pure proteins. Once isolated, several proteolytic digestion techniques have been optimized for the proteomic analysis of membrane proteins by mass spectrometry.

number of peptides compared to more specific protease. However, it does have a low amount of specificity for local sites in certain structural motifs as mentioned by Speers et al. [69].

Another protease that has been well characterized for the analysis of membrane proteins is elastase. Transmembrane (TM) proteins from *Halobacterium salinarium* purple and *Corynebacterium glutamicum* membranes were analyzed by a new proteolytic protocol developed by Rietschel et al. [133]. Their use of elastase with methanol resulted in a significant increase in the number of TM peptides. A significant portion of peptides (6577) were identified from *Halobacterium* and *Corynebacterium* [133]. There results revealed that elastase has an absolute specificity greater than 82% for cleavage of Ala, Val, Leu, Ile, Ser and Thr when at the P_1 position (i.e. the residue before the cleaved peptide bond) [133]. The authors concluded that elastase is a well suited enzyme for the proteomic analysis of membrane proteins and is able to circumvent the problems associated with tryptic digestions. Pepsin has been shown to be a valuable protease for the proteomic study of membrane proteins [120,135]. In recent studies, Rietschel et al. have optimized an existing (in solution) pepsin digest protocol for the proteomic analysis of the 7-transmembrane helix H+-ATPase bacteriorhodopsin (BR) from *Halobacterium* purple membranes (PM). The PMs were solubilized in a Trifluoracetic acid/methanol (TFA/MeOH) solution prior to digestion [134]. Then 1% w/w pepsin was added and the solution was incubated for 16 h at 22 °C [134]. A control reaction was prepared with 10% formic acid (FA) and 200 μL of water. Spectra from the control reaction displayed significantly reduced intensity and S/N values compared to the TFA/MeOH reaction [134]. The specificity of pepsin was also assessed. They indicate that the most frequently carboxy-terminal cleaved residue is Leu followed by Ala, Phe and Glu. The authors conclude that pepsin has considerable potential for the proteomic analysis of membrane proteins and can be considered an alternative for their protocol [134].

Non-specific proteases have been used successfully for the proteomic analysis of membrane proteins, as previously mentioned. However, a significant number of peptides generated from non-specific proteases are hard to predict because of the random location of positive charges, which direct charge fragmentation. Charge-directed fragmentation can be manipulated by the addition of reagents that specifically modify the N-terminal and specific side chains. This will increase the number of b- or y-ion series fragments. An N-terminal modification procedure based on nicotinylation for duplex isotopic labeling of digested proteins has been previously shown [136]. Adding a basic group at the N-terminal of peptides has been shown to increase the b-ion series relative abundance [137,138]. Janson et al. have shown that N-terminal nicotinylation of a peptide from a proteinase K digestion shows a complete b-ion series compared to the unmodified peptide that showed a weak y-ion series and an incomplete b-ion series [135]. In addition, a modified peptide digested with pepsin had a much improved spectrum when compared to the unmodified peptide [135]. The authors also discussed the effect of nicotinylation on database searching.

Mascot was used to assess the effect of N-terminal nicotinylation on MS/MS database search identifications and scores [135]. Because succinylation of lysine residues is a

consequence of the modification process, the overall charge state of the peptides is affected. Using Mascot, the authors determined that the effect of nicotinylation and succinylation caused the number of peptides to decrease when selecting 2+/3+ ions, and increase when selecting 1+/2+ ions [135]. They also concluded that the modification process significantly increased the number of peptide matches and scores when selecting 1+/2+ ions compared to the unmodified peptide [135].

3.6. *Quantitative proteomic analysis of membrane proteins*

For the purposes of this review, the culmination of the methods developed for shotgun proteomics, quantitative proteomics, and membrane protein analysis is the quantitative proteomic analysis of membrane proteins. This was once a very challenging task prior to the extensive method development in proteomics in general over the past several years. Nearly all of the methods described in this review have been used in a quantitative proteomic analysis of membrane proteins. MudPIT has been used to analyze changes in the *Saccharomyces cerevisiae* membrane proteome under different nutrient conditions [53] and changes in *C. glutamicum* membranes on alternative carbon sources [139,140]. SCX fractionation followed by RP/MS/MS was used to analyze cell surface proteins from human embryonic stem cells [141] and the *Escherichia coli* membrane proteome [142]. Examples of quantitative membrane proteome analyses using IEF approaches include the analysis of rat Golgi [143], drug resistance in small cell lung cancer [144], and evaluation of the IPG-IEF method [145]. GeLC/MS/MS approaches have been used to identify β-secretase subunits [146], macrophage raft changes in response to lipopolysaccharide [147], membrane proteome changes between self renewing and differentiating human embryonic stem cells [148], new substrates for the MARCH9 transmembrane E3 ligase [149], and *Bacillus subtilis* in response to salt stress [150]. Finally, blue-native PAGE was used to analyze changes in yeast mitochondrial membrane proteins in response to aerobic and anaerobic conditions [151] and erythrocyte membrane proteomes [152]. In all likelihood, given the excellent technologies for the analysis of membrane proteins by shotgun proteomic approaches, quantitative proteomic analysis of membrane proteins should become more and more common.

4. Future perspectives

Shotgun proteomics has been proven to be a valuable tool for identifying new large or small proteomes and protein complexes, which has allowed for the identification of previously unknown protein–protein interactions. However, a challenge still remains for the proteomic analyses of membrane proteins. Providing that membrane proteins make up 30% of naturally occurring proteins, and because they are implicated in a wide variety of functions, the ability to perform shotgun proteomic analyses is crucial for understanding their function. However, due to their hydrophobic nature, characterization of membrane proteins by mass spectrometry has been a challenge. Over the years many techniques have been developed for the isolation and separation of membrane proteins for proteomic analyses as discussed herein. Future advances in technology will also play a critical role for the field of proteomics. New instrumentation that performs faster scanning speeds and higher mass accuracy will also allow for more peptide identifications and ultimately higher proteome coverage, which will facilitate the analysis of membrane proteins.

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