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Review

Integral membrane proteins in proteomics. How to break open the black box?

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ABSTRACT

Integral membrane proteins (IMPs) are coded by 20–30% of human genes and execute important functions – transmembrane transport, signal transduction, cell-cell communication, cell adhesion to the extracellular matrix, and many other processes. Due to their hydrophobicity, low expression and lack of trypsin cleavage sites in their transmembrane segments, IMPs have been generally under-represented in routine proteomic analyses. However, the field of membrane proteomics has changed markedly in the past decade, namely due to the introduction of filter assisted sample preparation (FASP), the establishment of cell surface capture (CSC) protocols, and the development of methods that enable analysis of the hydrophobic transmembrane segments. This review will summarize the recent developments in the field and outline the most successful strategies for the analysis of integral membrane proteins.

Significance: Integral membrane proteins (IMPs) are attractive therapeutic targets mostly due to their many important functions. However, our knowledge of the membrane proteome is severely limited to effectively exploit their potential. This is mostly due to the lack of appropriate techniques or methods compatible with the typical features of IMPs, namely hydrophobicity, low expression and lack of trypsin cleavage sites. This review summarizes the most recent development in membrane proteomics and outlines the most successful strategies for their large-scale analysis.

Contents

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1. Introduction

Cellular membranes provide an essential physical interface between individual subcellular compartments, and between the cell and its environment. Composed of proteins, phospholipids and glycolipids, cellular membranes play a critical role in cell function and survival by spatially restricting chemical and biochemical processes and defining cell borders. Integral membrane proteins, i.e. proteins that cross the phospholipid bilayer, are coded by roughly 25% of human genes [1] while representing circa 7–8% of the overall cellular protein mass in human cells [2].

Integral membrane proteins (IMPs) function as important transporters, channels, receptors, and enzymes, responsible for signal transduction, regulatory processes and cell-cell and cell-environment interactions. These roles make IMPs enormously attractive targets for therapeutic interventions. In fact, approximately half of the currently approved drugs in human medicine target IMPs [3]. The recent revolution in the development of therapeutic antibodies against surface plasma membrane proteins further augmented the wide interest in IMPs. However, our knowledge of the structure, function and expression dynamics of IMPs is still limited, mostly because of their adverse physico-chemical properties and low expression levels. Based on their structure, IMPs can be characterized as alpha-helical or beta-barrel proteins. Since beta-barrels are a minor component of mammalian genomes, restricted to several proteins of bacterial origin present in the mitochondrial membrane and can be studied by conventional approaches, this review will further discuss only the hydrophobic alpha-helical proteins. Similarly, monotopic membrane proteins, i.e. molecules that are attached or anchored to the membrane but do not traverse the bilayer, will not be discussed here.

Alpha-helical IMPs are amphipathic – composed of hydrophilic extramembrane segments and one or more hydrophobic alpha-helical segments of 20–30 amino acids spanning the phospholipid bilayer. It is this amphipathy that renders IMPs difficult to solubilize and makes membrane proteomics so challenging. Due to their "split personality" and low expression levels, IMPs are underrepresented in conventional bottom-up proteomic analyses, which generally favor soluble, abundant and easy-to-digest proteins and peptides. [4,5]. In addition to their low abundance and relative hydrophobicity, a third adverse feature of IMPs exists – low digestibility with trypsin– since the hydrophobic alpha-helical segments are poor in the charged lysines (K) and arginines (R) that are the targets for trypsin. Furthermore, the exposed hydrophilic extra-membrane segments are often of limited length and may not provide enough tryptic peptides for identification, despite being adequately rich in tryptic cleavage sites (see Fig. 1). For more information on IMP structure and their alpha helices, several high quality reviews can be recommended [6–8].

This review will summarize recent developments in the proteomics of mammalian IMPs, namely the progress in sample preparation steps preceding LC-MS analysis, and outline the most successful strategies to date regarding the number of identified IMPs and their enrichment. We will therefore deal mostly with the different strategies of solubilization and digestion of membrane samples. We also do not address the conventional 2-DE technology, as it has been largely abandoned and demonstrated to be unsuitable for the analysis of mammalian hydrophobic IMPs in complex mixtures. This incompatibility has several reasons, the major one being the low solubility of IMPs during isoelectric focusing (IEF). For detailed information on 2-DE applications in membrane proteomics, two excellent reviews can be highly recommended [9,10].

In proteomic publications, the numbers of identified IMPs are usually reported either according to their proper Gene Ontology (G.O.) annotation or, more stringently, as numbers of proteins containing at least one predicted transmembrane segment. Experimental evidence on the topology of IMPs is quite limited, but topology prediction algorithms provide fast, though potentially inaccurate information on probable IMP topology. Early methods for topology prediction were based solely on the identification of hydrophobic stretches of 15–25 amino acid residues in the protein sequence. Later, with the discovery of the "positive-inside" rule [11], predictions were further refined by enabling the correct orientation of TM segments. Current prediction methods use various algorithms ranging from the "sliding window across the sequence" to more advanced artificial neural networks, support vector machines, hidden Markov models and dynamic Bayesian networks (for review see [1,12–15]). Among the difficulties in topology prediction are hydrophobic signal sequences that are similar to N-terminal TM segments, kinks in the TM segments, and short re-entrant segments. Some of prediction methods are, however, capable of dealing even with these obstacles. Several of the algorithms also use evolutionary information by introducing multiple sequence alignments, or refine the prediction with incorporation of preexisting knowledge of the topology of some regions of the sequence. The very recently introduced CCTOP prediction web server integrates 10 different topology prediction methods [16]. In this review, we have made an effort to report the numbers of IMPs identified in the referenced publications based on the prediction of TM segments. In several cases, where such information was not available in the publication, we used the original published data and recalculated the proportion of IMPs with the TMHMM algorithm based on the hidden Markov models [17].

The field of membrane proteomics has changed dramatically in the last decade. Aside from the massive improvement in mass spectrometry (MS) instrumentation, the advent of MS-compatible detergents, filter assisted sample preparation (FASP) and surface capture protocols have markedly diversified our proteomic tool-box and opened the way toward understanding the proverbial black box of the membrane proteome.

2. Enrichment of membrane material

In general, two basic strategies in membrane proteomics exist. Membrane proteins can be either targeted as whole molecules, or alternatively, a "divide and conquer" approach can be used, aiming separately or exclusively at their hydrophilic (extramembrane) or hydrophobic (transmembrane) segments. Regardless of the strategy, membrane enrichment is an essential, and usually the first, step in both workflows. The enrichment of membrane material is almost always performed by centrifugation, ranging from one-step medium-speed crude membrane sedimentation to multistep isolation involving a density gradient or

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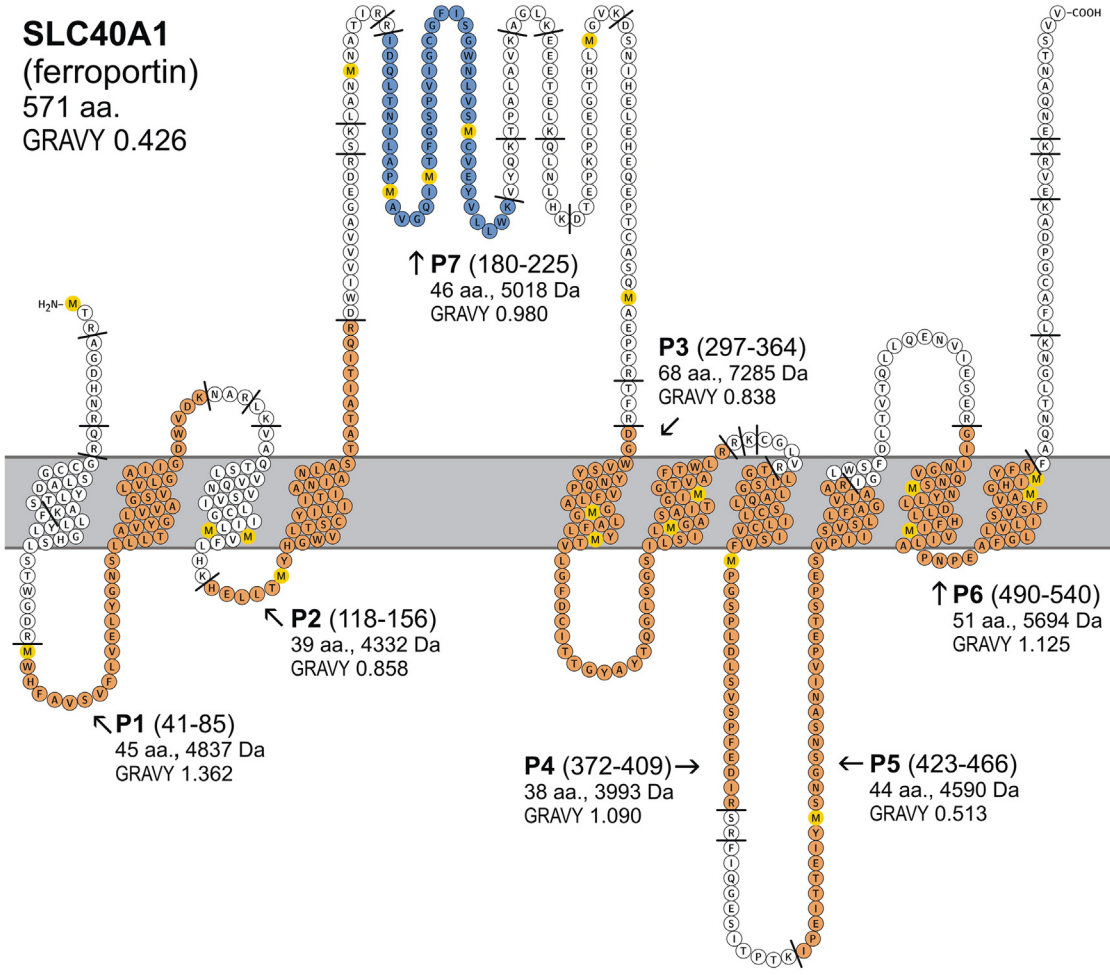

Fig. 1. The problematic digestion of IMPs with trypsin. An example of an integral membrane protein with multiple transmembrane domains – the membrane iron exporter ferroportin (10 transmembrane segments, 571 amino acids, GRAVY score 0,426). Without solubilization of the protein, a theoretical digestion with trypsin would produce 11 peptides of length between 5 and 25 amino acids. A theoretical tryptic digestion of completely solubilized ferroportin (no missed cleavages) would generate only 2 additional peptides shorter than 35 amino acids. However, an entire 49% of the molecule sequence will be represented by 6 long transmembrane hydrophobic peptides (P1-P6, orange) ranging in length from 38 to 68 amino acids (MW from 3990 to over 7290) and high hydrophobicity (GRAVY scores from 0.51 up to 1.36). Such peptides may easily get lost during sample preparation and analysis. In addition to the critical transmembrane α-helices, even the extra-membrane (soluble) portion of the ferroprotin molecule contributes one long and hydrophobic segment (P7, blue, 5018 Da, GRAVY 0.98), further increasing the proportion of the ferroprotin sequence that could possibly escape detection. Graphics: http://wlab.ethz.ch/protter/

cushion ultra-centrifugation (reviewed in [18]) based on protocols established several decades ago. Plasma membrane enrichment via peeling by a cationic silica pellicle is a methodically interesting approach, which has not, however, found wider application [19].

No matter how complex the sedimentation strategies are, due to subcellular complexity and the large hydrophobic surface of membrane vesicles, isolated "membrane enriched" fractions are inevitably heavily contaminated by major cellular proteins, ribosomes, components of the cytoskeleton, proteins attached to membranes, and other molecules. In fact, these contaminants dominate MS spectra and hamper the analysis of underrepresented IMPs. To further enrich IMPs and strip the soluble contaminants and peripheral membrane proteins, isolated membrane fractions can be washed with aqueous high ionic strength buffers, typically using high pH sodium carbonate washes originally introduced by Fujiki in the 1980s [20]. In addition to the striping of peripheral proteins, the alkaline pH and high ionic strength of sodium carbonate stimulate opening of the membrane vesicles and releasing the entrapped contents, and alkaline carbonate washing is now routinely employed in membrane proteomics [21–23 and others]. In addition, high salt washes with 1–5 M NaCl, KCl or NaBr are sometimes added to the carbonate washes [19,24–28]. As an alternative to sodium carbonate treatment, membrane washes with an organic solvent, namely trifluoroethanol (TFE), have been also successfully tested [29]. It is evident, though, that no matter how vigorous and intensive such washing steps are, they only partially reduce the presence of non-membrane proteins in the sample, as the percentage of IMPs identified in isolated and carbonate-washed membrane fractions ranges from 20 to 60% of all identified proteins even after multi-step fractionation procedures. [21,25,30–32].

No significant developments in the membrane enrichment, in the classical sense, have been made in the last decade. However, a strategy for analysis of the plasma membrane proteome based on the affinity enrichment of surface proteins, known as Cell Surface Capture (CSC), has been developed and successfully applied in numerous studies. This method will be discussed in Section 5.1.

3. Solubilization and digestion

An enriched membrane fraction is the starting material in most proteomic workflows focusing on IMPs. Extracellular segments of IMPs with large extramembrane segments may, and often do, provide several peptides sufficient for protein identification even without membrane solubilization [21,33–35]. However, a significant proportion of an IMP molecule (and in some cases most of it) is safely buried in the phospholipid bilayer, and inaccessible to protease activity. The lack of solubility of IMPs not only prevents their complete digestion but also accounts

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Table 1
The most common detergents, organic solvents and chaotropes in membrane proteomics.

Compound	Advantages for membrane proteomics	Disadvantages
Detergents		
Sodium dodecyl sulfate (SDS)	-Efficiently solubilizes cell membranes and denatures all types of proteins -Can be removed using the FASP procedure	-Proteases do not tolerate concentrations of SDS higher than 0.1% -Even very low concentrations of SDS impair liquid chromatography and mass spectrometry -Removal procedures are either relatively laborious, inefficient or cause partial loss of the sample
Sodium dodecyl cholate (SDC)	-Compatible with trypsin in the concentration necessary for membrane solubilization (5% SDC) -Efficient removal by phase transfer or acid precipitation	-Lower solubilizing and denaturing ability compared to SDS
Acid-labile surfactants	-Rapid detergent removal by acid cleavage	-Expensive -Loss of hydrophobic peptides after acid cleavage and precipitation (RapiGest)
Organic solvents		
Methanol, trifluoroethanol	-60% methanol (or 50% TFE) solubilizes membranes and IMPs -Trypsin remains partially active in 60% methanol (or 25% TFE) -5–15% TFE can be used for removal of membrane-associated proteins from membrane surfaces -Easy evaporation prior to LC	-Trypsin activity and specificity are severely lowered in 60% methanol
Formic acid	-Effectively solubilizes membranes, hydrophobic proteins and peptides -Compatible with chemical cleavage using cyanogen bromide	-Incompatible with most proteases -May cause formylation and hydrolysis of proteins and peptides
Chaotropes		
Urea, guanidine hydrochloride	-6–8 M urea can be used to denature extra-membrane parts of IMPs in combination with certain proteases (Lys-C, Glu-C) -Efficient removal of both guanidine and urea prior to LC/MS using common desalting methods	-Does not solubilize membranes nor IMPs -Incompatible with trypsin at concentrations needed for protein denaturation (6 M guanidine, 6–8 M urea) -Urea may cause protein modifications at elevated temperatures

for protein precipitation, aggregation and non-specific adhesion to laboratory plastic. Therefore, solubilization of the membrane material is the cornerstone of success in membrane proteomics. The solubilization step is particularly important for the release of peptides resulting from a cleavage in extramembrane loops of IMPs with multiple transmembrane segments. Chaotropes, organic solvents and especially detergents may assist in this task to various extents (Table 1).

3.1. Detergents

Detergents are amphipathic molecules mimicking the properties of the membrane phospholipids, including the assembly of micelles. That makes them enormously useful in membrane disintegration and protein solubilization. However, detergents differ greatly in their solubilization power and denaturing effects. Also, depending on their characteristics, detergents may inactivate trypsin and other proteases, stick to hydrophobic surfaces, interfere with chromatographic separation and/or suppress peptide ionization and contaminate mass spectrometers. Therefore, significant effort has been invested into the advancement of methods for detergent removal prior to digestion or LC-MS analysis, and into the development of new detergents without such adverse effects.

3.1.1. SDS

Sodium dodecyl sulfate (SDS), a linear chain strong ionic detergent, is highly effective in the solubilization of membranes and membrane proteins as well as in protein denaturation [36]. Trypsin activity is limited in even 0.1% SDS, however, and much lower SDS concentrations can cause a reduction in the separation power of liquid chromatography (LC) and hamper peptide ionization during MS analysis [37–41]. Therefore, SDS must be removed prior to protein digestion or LC-MS/MS. To remove SDS from a membrane sample, ion-pair extraction using a mixture of triethylamine, acetone, acetic acid and water can be used [42]. Precipitation of SDS by potassium chloride is also effective and possibly more convenient [43]. Alternatively, protein precipitation with organic solvents, namely TCA [44,45], acetone, or chloroform/methanol/water [46] is an effective, simple and inexpensive way to deplete SDS with sufficient protein recovery.

Similarly to traditional in-gel protein digestion, a complex membrane sample containing SDS can be briefly electrophoresed on conventional SDS-PAGE, SDS extracted from the gel slice, and then proteins in the gel digested and peptides extracted [19,22,47–50]. In a less laborious alternative, the SDS-solubilized sample is simply mixed with a small amount of the acrylamide solution prior to its polymerization [32,51,52]. The in-gel digestion trypsin-based approach solves the problem of SDS; unfortunately, however, its applicability for the analysis of IMPs, especially IMPs with multiple transmembrane segments, is limited, since long hydrophobic peptides resist extraction from acrylamide gels [22].

Another methodically distinct way of SDS depletion from a complex sample is based on the covalent capture of proteins. Magnetic nanobeads coated with tresyl-functioned PEG covalently bind free amino groups of proteins from SDS-solubilized membranes. The captured proteins can then be washed and digested. A recent application of this approach in the analysis of a liver microsomal fraction enabled the identification of >1500 IMPs, representing roughly a quarter of all proteins identified in the study [53]. SDS depletion using SCX chromatography has also been explored [54] and found efficient in samples where SDS concentrations exceed its critical micellar concentration [55]. More importantly, a successful detergent removal on a desalting size-exclusion column with the assistance of 8 M urea [27] led to the introduction of filter assisted sample preparation (FASP) - an elegant method of sample clean-up and digestion [56].

FASP allows the depletion of detergents (or generally any low molecular weight soluble compounds) including SDS from a complex sample by centrifugation through an ultrafilter with a 10–30 kDa cut-off combined with washes with 8 M urea buffer. Further buffer exchange and a direct on-filter sample digestion may follow the washing step. This was demonstrated to be superior to the in-solution digestion approach in terms of protein sequence coverage, number of protein identifications and the absence of bias against hydrophobic proteins [56]. The applicability of the FASP workflow to membrane proteomics was clearly demonstrated in an analysis of a mouse hippocampal membrane fraction using a double on-filter digestion with Lys-C followed by trypsin that allowed the identification of over 1600 IMPs [30]. In combination with a complex multi-step sample processing procedure, GELFrEE (gel eluted liquid fraction entrapment electrophoresis), the application of FASP later enabled the identification of 2090 IMPs from a membrane fraction of human leukemia cells [57]. Recently, FASP facilitated the identification of over 300 IMPs in an analysis of technically-challenging membrane microdomains of a human renal carcinoma [58].

A modification of the FASP method with lectin-affinity capture led to development of glyco-FASP [59], enabling the selective enrichment of

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glycosylated peptides from IMP extramembrane domains of the plasmatic membrane, lysosomes and endosomes (see more in Section 5.3.).

Being widely adopted and incorporated into various workflows, the elegantly simple FASP has changed the methodological repertoire of current proteomics. As evidenced above, FASP combined with a strong detergent, namely SDS, has enabled the identification of up to 1000–2000 IMPs in a single study.

3.1.2. Sodium deoxycholate

Although SDS has been the detergent of choice for the solubilization of membrane proteins, alternatives have been sought and evaluated. Sodium deoxycholate (SDC) is an ionic detergent with a steroidal hydrophobic part and a charged carboxyl group. SDC facilitates the digestion of hydrophobic proteins through efficient denaturation, although compared to SDS, cholates possess lower denaturing and solubilizing ability [60,61]. One clear advantage of SDC is its high compatibility with trypsin, which tolerates SDC concentrations up to 5–10% [61,62].

Similarly to SDS, SDC must be removed from the peptide sample before MS analysis. It can be effectively depleted by phase transfer into a water-immiscible solvent, namely ethyl acetate [61]. SDC has been successfully applied to the analysis of membranes of human HeLa cells, resulting in the identification of a total of 1450 proteins, of which 512 (35%) were IMPs [61]. Later, an analysis of membrane samples from human breast tumors solubilized by SDC led to the identification of 7095 proteins including 1977 (28%) IMPs, with at least one transmembrane alpha helix predicted by TMHMM [63]. SDC also enabled the identification of 5556 proteins including 1567 (28%) IMPs in human colorectal cancer samples [64]. SDC can be alternatively removed by acid precipitation in aqueous buffer using 0.1–2% trifluoroacetic acid or formic acid followed by centrifugation [62,65]. Side-by-side comparisons of the phase transfer and acid precipitation of SDC have produced several conflicting reports. Lin et al. [66] showed that both strategies of SDC removal led to a loss of peptides, and this loss was more pronounced in the phase transfer method. Others have demonstrated that acid precipitation is more reproducible [67,68], as it does not require the potentially problematic removal of the aqueous phase. On the other hand, León et al. [69] documented higher sequence coverage in samples undergoing phase transfer compared to acid precipitation.

SDC has also been repeatedly evaluated side-by-side with other detergents including SDS and acid-cleavable RapiGest (see below), with SDC found to be superior for the solubilization and digestion of IMPs with trypsin [69,70]. In general, enabling the identification of 500–2000 IMPs, SDC has firmly established its potential for membrane analysis along with the traditional SDS.

3.1.3. Acid-labile surfactants

Acid-labile surfactants (ALS) have been developed relatively recently to avoid or simplify the detergent removal prior to LC-MS. As the name suggests, these detergents are cleaved by a low-pH environment at elevated temperature. Their hydrophobic part becomes water-immiscible, and forms an easily-removable precipitate, while the remaining part of the molecule is LC- and MS-compatible. RapiGest™ was the first ALS to reach wider attention [71]. It structurally mimics SDS by having an ionic sulfonate moiety and hydrophobic undecyl chain, and effectively solubilizes membranes. Another important advantage of RapiGest is that it does not limit trypsin activity in concentrations up to 1% [71–74]. RapiGest was shown to be more powerful compared to urea in the solubilization of organelle-enriched fractions [72]. It was initially found to be slightly more efficient in comparison with SDS in regard to the number of identified proteins in the human MCF-7 cell line. [54] In the same study, analysis of an *E. coli* membrane fraction using RapiGest resulted in the identification of 1626 proteins, with about half being predicted IMPs. When an improved method for SDS removal was applied, both SDS and RapiGest allowed a comparable number of protein identifications in MCF-7 cells, with around 400 IMPs [55]. Several other ALSs have been developed and tested, such as 3-[3-(1,1-bisalkyloxyethyl)pyridin-1-yl]propane-1-sulfonate (PPS) [75], and more recently Progenta Anionic Acid Labile Surfactants I and II were found to be a good alternative to RapiGest [76].

The advantage of RapiGest, i.e. simplified detergent removal is, nevertheless, counterbalanced by its high price. In addition, a major complication of the RapiGest application in membrane proteomics can be the loss of the most hydrophobic peptides due to co-precipitation with the hydrophobic RapiGest fragment during its removal [61,71,77]. With the advent of FASP, which enables effective detergent removal, traditional, effective and cheap SDS, SDC or other detergents may prevail over their expensive acid-cleavable alternatives.

Several excellent reviews are available that give more detail and wider information on the available detergents and their application in the analysis of membrane proteins [60,78,79].

3.2. Organic solvents

Organic solvents have mainly been tested as an alternative to detergents. After the advent of FASP, which enables easy detergent removal, however, their importance as single agents for membrane protein solubilization and digestion will probably fade. Despite this, here we review the most commonly used.

3.2.1. Methanol and trifluoroethanol

To completely avoid the troubles with detergents interfering with LC separation or MS analysis, methanol has been exploited as an alternative for IMP solubilization and digestion. Trypsin has been shown to retain most of its activity in up to 20% methanol [80]; however, higher concentrations are needed to solubilize membranes. Trypsin activity is reduced to one-fifth in 60% methanol compared to aqueous buffer [81] and its specificity may also decrease [82]. Nevertheless, a detergent-free solubilization of an isolated membrane fraction in 60% methanol (assisted by sonication) followed by tryptic digestion in the same solvent enabled the identification of over 700 IMPs (including peptides from their transmembrane segments) in murine macrophages [83] and almost 500 membrane proteins (according to their GO annotation) in human epithelial cells [84].

Recently, however, a side-by-side comparison by Moore demonstrated that solubilization and digestion in 60% methanol is markedly inferior compared to 0.15% RapiGest or 1% SDC in a yeast membrane fraction. The use of detergents increased the total number of identified proteins and IMPs several-fold compared to methanol. [70] As an alternative to methanol, 50% trifluoroethanol (TFE) has been evaluated as membrane-solubilizing agent for erythrocyte membranes. Despite the fact that TFE-solubilized membranes provided a lower number of identified IMPs compared to 60% methanol, the proteins and peptides released by TFE had markedly higher hydrophobicity, suggesting some potential of TFE as a complementary solvent for IMP analysis. [85]. TFE has also been employed in a methodically different setting – first, to release peripheral membrane proteins from isolated membranes (5% and 15% TFE wash) and then to solubilize delipidated membrane samples and assist trypsin digestion (25% TFE) in the analysis of a human NK-like cell line microsomal fraction. In this complex multi-step study including sample delipidation, 681 IMPs were identified. [29].

Although organic solvents may assist membrane solubilization, an extensive comparison of various solvents, detergents and chaotropes in the protein extraction and digestion of mouse brain samples demonstrated that detergent-based protocols including detergent removal by FASP significantly outperform organic solvents and chaotropes in the number of identified membrane proteins. The best detergent-based protocol enabled the identification of over 500 proteins, of which 29% were IMPs [86]. More recently, a similar side-by-side comparison of several detergents, two organic solvents, and chaotropes and their combinations confirmed the superiority of detergents over organic solvents and chaotropes in membrane analysis. The same study, however,

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pointed out the advantage of the additive effect of an organic solvent (acetonitrile), a chaotrope (guanidine) and an MS-compatible detergent used in combination. [76].

3.2.2. Formic acid

Concentrated formic acid (FA) is an excellent solvent for the solubilization of membranes and hydrophobic proteins and peptides. Significant downsides to FA, however, are its incompatibility with trypsin activity and tendency to generate uncontrolled damage to protein samples, like D-P bond cleavage [87] and protein formylation [88]. However, these modifications can be prevented by working at low temperatures [46].

Despite being incompatible with trypsin, FA has found its use as an alternative to SDS in the solubilization of membrane samples [89] and precipitated protein pellets [46], as a solvent for the extraction and MS-analysis of long hydrophobic peptides from acrylamide gels following their in-gel digestion [44], as well as a solvent for the digestion of membrane material with pepsin [90]. Most importantly, FA can be used for chemical cleavage of hydrophobic peptides with cyanogen bromide (CNBr) [24,91]. This will be further discussed in Sections 4.1.6. and 5.2.

3.3. Chaotropes

Although less potent in membrane protein solubilization compared to detergents and organic solvents, chaotropes are sometimes used in proteomics for the disruption of protein-protein interactions, denaturation, and maintaining the unfolded state of proteins. Urea and guanidine hydrochloride are used most often. In contrast to detergents, these small molecules do not interfere significantly with LC/MS. If desired, urea and guanidine can be removed prior to MS by common desalting techniques. The optimal concentrations of guanidine hydrochloride or urea needed for denaturation of proteins are 6 M and 8 M, respectively, both far too high to be compatible with sufficient trypsin activity. Dilution to 1 M (guanidine) and 2 M (urea) concentrations before digestion of a protein sample with trypsin is needed. However, such a dilution may lead to protein refolding [74].

Although chaotropes do not fully extract IMPs from the membrane, urea has been reported to facilitate the digestion of their extramembrane parts [91–93]. However, a side-by-side evaluation of trypsin digestion efficiency in isolated membrane fractions showed no benefit of 2 M urea over ammonium bicarbonate buffer in terms of the number of the identified membrane proteins [25]. Similarly, 1 M guanidine hydrochloride enhances the digestion efficiency of trypsin compared to ammonium bicarbonate buffer without additives [45,74] and its advantage compared to urea is also its chemical inertness [94]. Compared to detergents including ALS, the effect of chaotropes in membrane sample solubilization and digestion has been shown to be lower in terms of the number of identified proteins and peptides [25,74,76].

However, the role of chaotropes in membrane proteomics cannot be dismissed. The use of urea is inseparable from the use of endoproteases Lys-C and Glu-C, unique enzymes that tolerate up to 8 M urea. Digestion of a membrane sample with Lys-C in 6–8 M urea before dilution of the chaotrope and sample re-digestion with trypsin has been shown to improve IMP identification [25] and has become one of new standards in sample preparation (see more in Section 4.1.1).

4. Digestion of integral membrane proteins

Even the best solubilization strategy cannot increase the number of identified IMPs, nor improve their sequence coverage, without the production of peptides of a size and hydrophobicity compatible with current bottom-up technologies. Trypsin is undoubtedly the optimal protease for standard soluble protein sequences containing advantageous distributions of arginine and lysine residues [95]. However, traditional trypsin-centric strategies may not be sufficient for unlocking the secrets of the phospholipid bilayer, and the analysis of IMPs may require other strategies. Arginines and lysines, although sufficiently frequent in extracellular soluble segments of plasma membrane, may be sterically inaccessible to trypsin due to extensive glycosylation. [25,96]. Moreover, the size of trypsin-cleavable hydrophilic extramembrane portions of IMPs varies from conveniently large extracellular domains down to very short terminal or loop segments that may not provide enough sequence information for unequivocal protein identification. Most importantly, the well-established scarcity of charged lysine and arginine in hydrophobic transmembrane segments, formed preferentially by nonpolar and polar uncharged amino acids, is the most critical obstacle to effective trypsin use in membrane proteomics [97,98]. Trypsin-generated peptides including one or more transmembrane alpha-helical segments are inevitably large (30+ amino acids) and highly hydrophobic, as peptide hydrophobicity seems to be to some extent a function of their length [97]. Such peptides may readily adhere to plastic surfaces and get lost during sample preparation, may be retained on LC columns, or may not be detected by current MS instrumentation. The problem of long hydrophobic peptides resulting from trypsin cleavage is demonstrated in Fig. 1. A theoretical and complete (no missed cleavages) digestion of the fully solubilized IMP ferroportin demonstrates that half of the protein sequence is represented by 6 problematic transmembrane hydrophobic peptides with MW up to 7290 and high hydrophobicity as determined by their GRAVY score [99].

Some of the abovementioned complications of trypsin use in the analysis of IMPs may be overcome or at least limited: for instance, inclusion of a deglycosylation step prior to proteolytic digestion can lead to an increase in the number of identified IMPs [25]. Similarly, detectability of shorter trypsin-generated hydrophobic peptides by MS can be enhanced by chemical modifications leading to their increased solubility, such as the modification of methionines by oxidation [97]. However, such improvements are largely negligible, and a better solution may be to look for an alternative cleavage strategy. Such as strategy may be sought among other proteases, including specific, semi- and non-specific proteases, or among chemicals enabling peptide cleavage. Alternatively, hydrophilic and hydrophobic segments of IMPs can be targeted separately, with each requiring different strategies and tools.

4.1. The neglected world beyond trypsin

Trypsin has long been the gold standard in proteomics. Its clear dominance can be reflected in the number of available tryptic peptide datasets in databases. Enzymes other than trypsin account for only 4% of the data available in the Global Proteome Machine Database (GPM DB). Of the non-tryptic data, Lys-C has been the most significant contributor [100]. In the next paragraph, we will review the application of proteases other than trypsin in the analysis of IMPs, including the specific proteases Lys-C and Glu-C but also several semi-specific or nonspecific proteases (see also Table 2).

4.1.1. Lys-C and Glu-C

The major advantage of the bacterial serine endoprotease Lys-C (which is specific for the C-terminal peptide bond after lysine) is that it retains its activity in urea concentrations up to 8 M and enables the specific pre-digestion of hydrophobic samples in the presence of a chaotrope. This facilitates a partial solubilization and digestion of membrane proteins before urea removal and a final re-digestion with trypsin. This sequential strategy has been employed and shown to be beneficial in several membrane proteomics analyses, either in solution [34,35,52, 92,101,102 and others] or in a FASP-based arrangement [30,103]. In both cases, the advantage is that urea-denatured proteins are more prone to digestion, and after necessary dilution of the sample, this pre-digestion limits sample aggregation and facilitates the final digestion with trypsin.

The combination of detergent removal, pre-digestion with Lys-C in urea followed by final digestion with trypsin has established a workflow

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Table 2
Summary of proteases and chemicals with high potential for analysis of IMPs.

Enzyme/compound	Properties	Strengths	Weaknesses
Trypsin	-Cleaves C-terminally to Arg and Lys	-Generates doubly positively charged peptides -Efficient ionization of peptides, compatibility with SILAC -Tolerance to some organic solvents	-Arg and Lys in hydrophobic segments are rare – long hydrophobic peptides escape identification -Low tolerance to detergents and chaotropes
Lys-C	-Cleaves C-terminally to Lys	-Active in high concentrations of urea – allows efficient denaturation, suitable for "shaving-off" approaches	-Lys in hydrophobic segments is rare – long hydrophobic peptides escape identification -Incubation in sub-optimal temperature in the presence of urea to prevent carbamoylation
Glu-C (V8 protease)	-Cleaves C-terminally to Asp and Glu	-Active in high concentrations of urea – allows efficient denaturation, suitable for "shaving-off" approaches	-Asp and Glu in hydrophobic segments are rare – long hydrophobic peptides escape identification -Incubation in sub-optimal temperature in the presence of urea to prevent carbamoylation
Chymotrypsin	-Cleaves C-terminal to Phe, Tyr and Trp, to a lesser extent to Leu and Met	-Cleavage sites present in hydrophobic segments	-Not an entirely specific protease -Generates long hydrophilic peptides that escape identification
Elastase	-Cleaves preferably C-terminally to small uncharged amino acids, and to a lesser extent to other amino acids	-Allows cleavage in hydrophobic transmembrane segments	-Nonspecific: generates a complex mix of multiply-overlapping peptides
Pepsin	-Cleaves preferably C-terminally to Tyr, Phe, Trp, Leu, and to a lesser extent Ala, Gly.	-Active in the presence of formic acid, which is a good solvent for hydrophobic proteins	-Nonspecific: generates a complex mix of multiply-overlapping peptides
Proteinase K	-Nonspecific, but preferentially C-terminal to aliphatic and aromatic hydrophobic amino acids	-Attenuated at high pH – generates favorable lengths of peptides -In combination with sodium carbonate buffer good for "shaving-off" approaches	-Nonspecific: generates a complex mix of multiply-overlapping peptides
Cyanogen bromide	-Cleaves C-terminally to met, converts to homoserine or homoserine lactone	-Sequentially specific to hydrophobic amino acid occurring in transmembrane segments -No sterical hindrance, does not produce missed cleavages	-High toxicity

that has become almost a standard in membrane proteomics. Lys-C has been included in the most successful membrane proteomics studies. Among those certainly deserving mention are an analysis of mouse hippocampal membranes resulting in the identification of over 1600 IMPs [30], a study of human breast tumors that resulted in the identification of 7095 proteins including 1977 (28%) IMPs [63], and an analysis of a liver microsomal fraction with >1500 identified IMPs [53].

It should be kept in mind, however, that high concentrations of urea can lead to the carbamoylation of primary amino groups in the sample when exposed to temperatures above 30–40 °C for prolonged periods of time [104]. For this reason, urea-assisted Lys-C digestions are usually carried out at 30 °C or below. Although the inclusion of Lys-C in urea facilitates the solubilization and digestion of membrane proteins, especially of their extramembrane domains, it does not solve the existing problem of long transmembrane lysine- and arginine-less segments.

Glu-C (alias V8 protease) from *Staphylococcus aureus* also retains its activity in 8 M urea but cleaves peptide bonds on the COOH terminal side of either Glu or Asp. Since these charged amino acids are not frequent in transmembrane segments, Glu-C offers only limited advantages over Lys-C. Dormeyer et al. compared the performance of Glu-C with Lys-C in a sequential digestion in 8 M urea, followed by trypsin digestion. Both enzymes produced a similar percentage of IMPs among the identified proteins, but the use of Glu-C resulted in a lower overall number of identified proteins [25].

4.1.2. Chymotrypsin

Chymotrypsin cleaves at C-terminal peptide bonds following the large aromatic amino acids phenylalanine (F), tyrosine (Y) and tryptophan (W), and also with lower efficiency after leucine (L) and methionine (M). These amino acids occur relatively frequently in the hydrophobic transmembrane segments of IMPs. Its specificity for F, W and Y was reported to increase in the presence of organic solvents [80]. An in silico proteome analysis assessing optimal cleavage conditions for IMPs of eukaryote origin suggested that chymotrypsin in combination with trypsin would radically (100-fold) lower the occurrence of large peptides (>4 kDa), while the use of chymotrypsin alone was predicted to result in higher sequence coverage of IMPs by peptides of appropriate MWs between 0.6 and 4 kDa compared to trypsin [105].

Despite its theoretical advantages, chymotrypsin has been used in only a limited number of studies. Simultaneous chymotrypsin plus trypsin digestion in 60% methanol was tested on *Corynebacterium glutamicum* membranes and resulted in the identification of 267 IMPs. [24]. The number of identified IMPs was later increased to 297 when this procedure was modified by the addition of high salt washes and dextran-PEG phase partitioning [106]. For such a simple prokaryotic organism, three hundred IMPs represent a substantial fraction of its membrane proteome.

Recently, sequential digestion of yeast membrane fractions with trypsin-chymotrypsin in tandem was shown to be inferior compared to trypsin alone [70]. While Dormeyer et al. confirmed the suitability of the chymotrypsin-trypsin combination for mammalian IMPs, they also reported that in comparison with sequential Lys-C-trypsin digestion in urea, chymotrypsin with trypsin allowed fewer overall identifications of IMPs [25].

4.1.3. Elastase

Elastase has received only limited attention in proteomics. This semi-specific protease cleaves at the C-terminal side of small neutral amino acids. Rietschel et al. compared the performance of porcine pancreatic elastase with trypsin on methanol-solubilized bacterial membranes. The two enzymes showed only a very limited overlap of identified proteins and a different representation of identified peptides: while trypsin covered over-loop segments of IMPs, elastase allowed the identification of a high number of transmembrane peptides [82]. Elastase's relative specificity was found to be 70% for the peptide bond following five amino acids (I, V, A, T, L, S) and 30% for the rest [82]. In general, the use of a protease with low specificity leads to the generation of short multiply-overlapping peptides of which only a fraction is positively charged. To address this drawback, TMT tags were used to label membrane samples digested by elastase in 60% methanol, allowing increased identifications of hydrophobic neutral and acidic cleavage products of elastase by MALDI-MS. [107].

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4.1.4. Pepsin

As a gastric enzyme, pepsin has its highest activity between pH 2–4, and has been shown to preferentially cleave C-terminally to the aromatic and hydrophobic residues Y, F, W and L and to a lesser extent after A and G, its specificity being pH dependent [108–110].

So far, this protease has been employed in only a limited number of studies. Pepsin was used for the analysis of rat liver microsome membranes solubilized with 90% FA and digested with immobilized pepsin after FA dilution. Out of 235 identified proteins, 39% were IMPs [90]. Golizeh et al. compared several strategies for the digestion of microsomal membrane samples, and demonstrated the advantage of sequential pepsin and trypsin digestion in markedly increasing the sequence coverage of IMPs compared to cleavage with trypsin only [45].

4.1.5. Proteinase K

Nonspecific proteinase K (which can digest proteins down to dipeptides) was used by Wu et al. to digest or "shave" the extramembrane domains of IMPs in a protocol named "high pH-proteinase K" (hppK). The use of an alkaline carbonate buffer during agitation enabled the removal of membrane-adhered proteins and promoted the opening of membrane vesicles, enabling digestion at both membranes surfaces. The "shaved-off" soluble extramembrane peptides generated by proteinase K were analyzed by LC-MS, and 454 proteins (representing 28% of 1600 total identifications) were predicted to be IMPs. [21] More importantly, the hppK method was later extended to also include analysis of the hydrophobic transmembrane segments that remain safely protected from the protease activity by the phospholipid bilayer [91]. To make the long transmembrane segments amenable to MS analysis, the authors used chemical cleavage of the peptides with CNBr. This approach introduced a unique method for analysis of the neglected hydrophobic transmembrane segments and will be covered in detail in Section 5.2.

The potential of semi- or nonspecific proteases for the analysis of IMPs, and namely of their hydrophobic segments, may seem obvious. However, it should be kept in mind that non-tryptic non-specific peptides are usually more difficult to identify than tryptic peptides. This can be attributed to their poorer ionization and fragmentation and to the fact the lack of defined termini markedly increases database search space, as more possible peptides fall within the precursor mass tolerance and increase the false positive rates [111]. The handful of endoproteases reviewed here and summarized in Table 2 represents only a minor fraction of the proteolytic enzymes currently available for protein digestion in proteomics. More information on the current protease repertoire can be found in a recent review by Tsiatsiani et al. [100].

4.1.6. Chemical cleavage with cyanogen bromide

In addition to the rich protease palette, non-enzymatic protein cleavage further diversifies our proteomic toolbox, as exemplified by cyanogen bromide. In acidic environments (originally 0.1 N HCl [112], but more efficiently in 70% trifluoroacetic acid [113] or formic acid [114]) CNBr selectively reacts with methionine residues and yields peptidyl homoserine or homoserine lactone and an aminoacyl peptide fragment. Reduced cysteine residues may also be subject to cleavage under these conditions, although this reaction is very slow and can be avoided by cysteine alkylation [112]. Compared to any endoprotease, CNBr treatment is very robust, with yields reaching 90–100% cleaved methionine sites. The exceptions are oxidized methionines, which remain uncleaved [115], and methionines followed by serine or threonine residues, where the cleavage efficiency is reduced [116].

CNBr has been proposed as an optimal complementary tool for the MS analysis of hydrophobic IMPs, because of its specificity for the methionine C-terminal peptide bond and the fact that methionine occurs at relatively convenient intervals, mainly in TM helices [97]. Results of an in silico proteomic analysis of the yeast membrane proteome suggested a combination of CNBr and trypsin as one of the methods of choice, as it lowers the occurrence of large (>4 kDa) peptides compared to trypsin used alone, and leads to one of the highest sequence coverages in the given 0.6–4 kDa window for the yeast membrane proteome of all tested combinations of proteolytic agents [105]. As can be demonstrated by a model IMP – ferroportin – methionines are present in 5 of its 10 transmembrane segments, and the theoretical application of CNBr would generate shorter peptides from the trypsin-generated hydrophobic segments P1–6 (Fig. 1).

Van Montfort et al. demonstrated that following standard in-gel trypsin digestion, in-gel cleavage with CNBr roughly doubled the sequence coverage of hydrophobic transmembrane segments, while the coverage of non-membrane segments of IMPs did not change [117, 118]. Similar results were also reported for CNBr-only or a sequential CNBr/trypsin in-gel digestion of bacteriorhodopsin compared to trypsin alone [42]. A sequential CNBr–Lys-C–trypsin digestion was employed by Washburn et al. on a yeast crude membrane fraction [119], and CNBr–trypsin sequential digestion was applied to *C. glutamicum* membranes by Fischer et al. [24], In both studies, the inclusion of CNBr led to the identification of satisfactory numbers of IMPs (with regard to the MS instrumentation then available).

CNBr requires an acidic environment for specific protein digestion [120]. The use of formic or acetic acid as solvents at high temperature enables the simultaneous cleavage with CNBr and acid hydrolysis of the protein, which preferentially leads to cleavage on both the N- and C-termini of Asp residues. This dual chemical cleavage was employed by Lee et al. in an analysis of a rat kidney membrane fraction. Dual digestion increased the number of identified membrane proteins compared to CNBr or acid cleavage alone. A combination of dual cleavage and subsequent re-digestion with trypsin further increased the number of identified membrane proteins [114]. However, the presence of acetic or formic acid at increased temperature may cause extensive protein acetylation or formylation, respectively.

The great potential of CNBr is partly counterbalanced by its toxicity, which must always be considered though not exaggerated. Since only minor amounts (typically <10 mg) are used for a typical cleavage reaction, working in a fume hood and properly disposing the toxic waste (including precipitated vapors from sample evaporation) should be sufficient to ensure safe work.

Despite the large potential for IMP analysis due to its specificity, compatibility with organic acids and the relative high occurrence of methionine in hydrophobic transmembrane segments, CNBr has been rather neglected. More recently, however, CNBr in combination with proteinase K [91] or trypsin [121] has paved the way toward more comprehensive analyses of integral membrane proteins including their transmembrane segments, as will be discussed in Section 5.2.

5. "Divide and Conquer" strategies

5.1. Hydrophilic extramembrane segments

The difficult-to-overcome obstacles of membrane proteomics mentioned above, arising namely from the amphipathic nature of IMPs, have led inevitably to the development of "divide and conquer" strategies that aim separately or exclusively at either hydrophilic (extramembrane) or hydrophobic (transmembrane) segments. The hydrophilic extra-membrane segments of IMPs provide the easier target. Here, membrane proteomics intersects with glycoproteomics.

5.1.1. Cell-surface-capture (CSC)

Exposed extracellular segments of plasma membrane (glyco)proteins are attractive targets for labeling and affinity capture. Using biotin (or other) labels with different protein-reactive groups, different peptide moieties can be targeted. Using primary amine-reactive labels such as sulfo-NHS-SS-biotin (containing a disulfide bridge for simple reductive elution) and sulfo-NHS-LC-biotin (containing a long chain linker), the N-termini and primary amine groups of accessible lysines are

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labeled [122]. More importantly, periodate-oxidated sugar moieties of surface glycoproteins can be labeled using hydrazide chemistry [123,124]. After solubilization and digestion, biotinylated (glyco)peptides from hydrophilic segments of exposed molecules can be affinity-isolated using streptavidin-coated beads. Isolated glyco-peptides can then be eluted using peptide-N-glycosidase F (PNGase F) and subjected to MS/MS analysis.

The initial use of sulfo-NHS-SS-biotin [125] and sulfo-NHS-LC-biotin [126] in analyses of cell surface proteins led to only moderate numbers of identifications and relatively low IMP enrichment. Wollscheid et al. enhanced the protocol for glycopeptide capture and established the use of these labels, namely biocytin hydrazide in an optimized procedure termed Cell Surface Capture (CSC) [127]. This method has been applied to the analysis of the plasma membrane proteome in cell cultures or primary cells to study various biological processes such as T-cell activation [127], the cell surface response to the induction of selected signaling pathways [128], and the response to retinoic acid stimulation in human leukemia cells [129], as well as to characterize induced pluripotent stem cells [130], study the druggability of glioblastoma cells [131], the physiology of primary adipocytes [132], surfaceome changes during the development of neural cells [133,134] and others. Some of these studies used the CSC method in a quantitative arrangement combined with SILAC [127], or label-free analysis [128,129,131–134].

Besides the biomedical reach of these works, from the technical perspective of membrane proteomics, this method has allowed an unprecedented enrichment of predicted IMPs reaching up to 90% [130] and up to 600 identified proteins with predicted transmembrane segments. The success of this method and the extensive number of plasma membrane proteome publications has given rise to a new term: surfaceomics or surfomics. The recently assembled "Cell Surface Protein Atlas" is a database of the surfaceomes of over seventy human and mouse cell types, containing 1500 human and 1300 mouse surface glycoproteins [135]. Despite the unprecedented IMP enrichment and high numbers of plasma membrane proteins identified, one should be aware of two limitations of the CSC method: it requires live cells, and preferentially targets the N-glycoproteins of the plasma membrane. One less obvious drawback may be the laboriousness of the method, which may explain the relatively limited number of laboratories currently adopting this promising technique.

5.1.2. SPEG

The requirement for live cells in the CSC protocol arises from the imperative for amine-reactive labels, where prevention of their penetration into the cell is essential [129]. In the more common cell surface (glyco)capture, the labeling of live cells enables a high enrichment of IMPs. However, glycosylation-targeted labeling is more or less specific for membrane and secreted proteins (soluble cytosolic proteins are only very rarely glycosylated) and can be modified for fresh or frozen tissues, as recently demonstrated by Liu et al. [136]. Solid Phase Extraction of formerly N-glycosylated Glycoproteins method (SPEG) uses the conjugation of oxidized sugars to hydrazide-coated beads [137,138] and has been used to identify glycoproteins associated with tumor aggressiveness in prostate cancer samples [136,139,140]. After extensive washing, glycopeptides were eluted from the hydrazide beads with PNGase F and subjected to SWATH-MS. This led to the identification of almost 900 glycoproteins, with 220 differentially expressed, of which 56% were predicted to be IMPs. [136].

5.1.3. Glyco-FASP

An alternative surface-oriented approach that combines the FASP method with lectin-affinity capture was introduced by Zielinska et al. [59]. Enrichment of glycosylated plasma membrane proteins from a whole cell lysate was achieved by the capture of SDS-solubilized glycoproteins on a lectin layer in an ultrafilter. After on-filter digestion, glycopetides remained captured by the lectin filter and were later released by PNGase. This strategy enabled the identification of 2352 glycoproteins in mouse tissues [59]. In a more recent study, Han et al. combined three FASP-based approaches including glyco-FASP, and identified 2360 IMPs in mouse tissue [141]. Deeb et al. used glyco-FASP in the analysis of human lymphoma cells, resulting in IMP enrichment (70%) and the identification of 925 IMPs [142]. Despite the impressively high numbers of identified IMPs, both N-glyco-FASP and SPEG have an identical limitation, namely exclusively targeting N-glycosylated peptides. Such peptides can be found not only in IMPs but also in secreted and potentially other non-membrane proteins. Other peptides and proteins are omitted by these methods, limiting complete descriptions of membrane proteomes.

5.2. Hydrophobic transmembrane segments

The attractivity of the accessible cell surface, advantageous specificity of glycosylation for membrane proteins and friendly nature of soluble domains are obvious. However, a significant portion of each IMP (in some cases most of the molecule) remains hidden deep in the phospholipid bilayer, inaccessible to most conventional methods and resistant to trypsin digestion. How can we effectively tap the treasure trove of information hidden in the phospholipid bilayer?

5.2.1. hppK-CNBr

A pioneering step in the analysis of the overlooked hydrophobic membrane-embedded segments of IMPs was taken by Blackler et al. [91], taking advantage of the important observation that the phospholipid bilayer effectively protects the transmembrane segments of IMPs from enzymatic proteolysis. In their hppK-CNBr (high pH, proteinase K) method, isolated membrane vesicles were "opened" by a sodium carbonate wash at high pH and low temperature, and treated with nonspecific proteinase K. The use of an alkaline carbonate buffer during agitation enabled the removal of membrane-adhered proteins and promoted the opening of membrane vesicles [20], enabling digestion at both membrane surfaces along with the unprotected extramembrane segments of IMPs [21]. The protease-treated lipid bilayer with the embedded protease-protected hydrophobic peptides was then further processed. As the membrane-embedded segments of IMPs are rather large and highly hydrophobic for optimal LC-MS analysis, they had to be re-digested. Shaved membranes were therefore solubilized in concentrated formic acid, and effectively re-digested with CNBr (which cleaves at methionine, see Section 4.1.6). The CNBr-cleaved samples were then delipidated by lipid precipitation in a diluted organic solvent and analyzed by LC-MS. Using this method, they identified 670 proteins in human HeLa cells. Of the 670 proteins 479 (72%) were IMPs. Importantly, two thirds of the identified IMPs were identified by peptides that overlapped with predicted transmembrane segments.

The pioneering hppK-CNBr method is not limited to plasma membrane proteins and can also be applied to frozen samples or tissue biopsies. Despite its high enrichment and the obvious potential for tapping the valuable information hidden in the phospholipid bilayer, the strategy has not attracted many followers. This is possibly because of the laborious multi-step workflow and application of less common cleavage strategies (non-specific proteinase K and CNBr), and potentially also the safety issues when working with toxic CNBr. Recently, our group has followed the original hppK-CNBr philosophy and introduced several modifications to the method, and applied it to the analysis of human lymphoma cells (see below).

5.2.2. hpTC

The use of non-specific proteinase K in the original hppK-CNBr protocol enables the efficient shaving of extramembrane protein material from both sides of membrane vesicles. However, the low specificity of proteinase K produces numerous overlapping peptides, leading to increased sample complexity and potentially decreasing the number of identified proteins. Moreover, the use of proteinase K precludes

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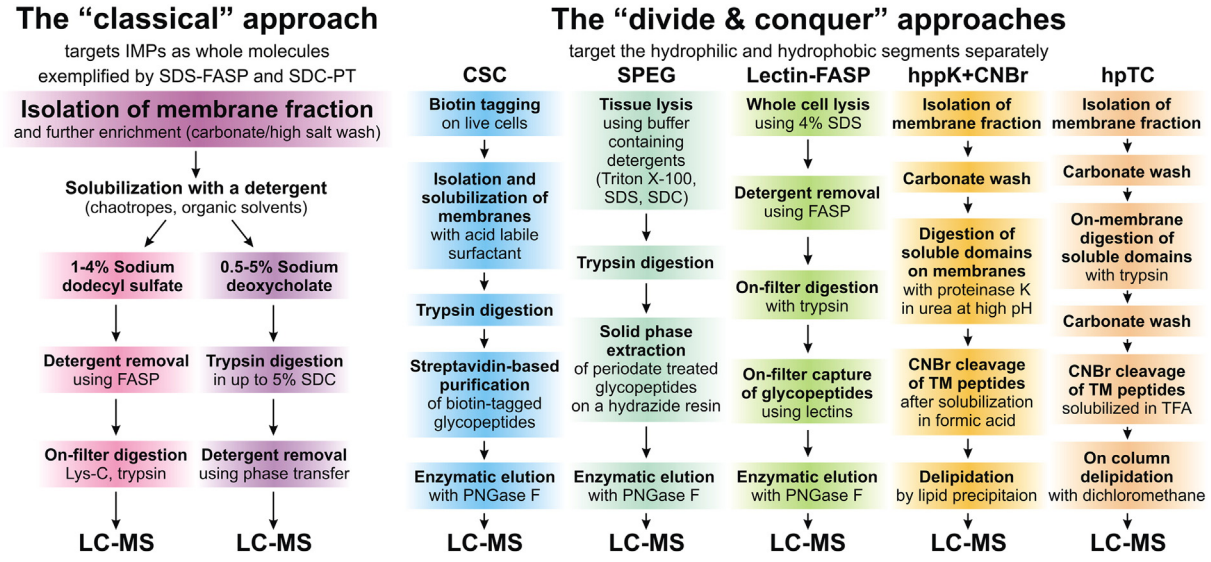

Fig. 2. The most successful strategies for proteomic analysis of IMPs. Two basic strategies in membrane proteomics can be defined. Membrane proteins are targeted either as whole molecules, or the "divide and conquer" approach can be used, aiming separately or exclusively at their hydrophilic (extramembrane) or hydrophobic (transmembrane) segments. The "classical" approach (left) is represented here by two most successful protocols, SDS-FASP and SDC–phase transfer removal (PTC). The "divide and conquer" approaches (right) target either hydrophilic domains of IMPs, as in the cases of CSC, SPEG and Lectin-FASP or hydrophobic transmembrane segments in the cases of hppK + CNBr and hpTC.

quantitative analysis using SILAC and potentially complicates label-free quantitation. Our group has modified the original method [121], in particular excluding an unnecessary ultracentrifugation step, replacing proteinase K with trypsin, and employing on-column sample delipidation with dichloromethane (a method originally devised for detergent removal [143]) instead of lipid precipitation in the aqueous-organic solvent used by Blackler et al. [91]. In reference to the original hppK method, we use the abbreviation hpTC (high pH, trypsin, CNBr).

This altered strategy enabled us to identify 1224 proteins in human lymphoma cells, including 802 (65.5%) IMPs with 1 to 16 transmembrane domains. Roughly half of the unique peptides belonging to IMPs overlapped with predicted transmembrane segments. Among the proteins identified were thirteen so-called "missing proteins", i.e. proteins with no previous evidence at the protein level. The introduction of trypsin instead of proteinase K eliminates the production of multiple overlapping peptides and increases the method's sensitivity and, most importantly, opens a new way toward combining this method with SILAC or label-free quantitation.

6. Conclusions

Aside from the rapid development of MS instrumentation, several methodical innovations and novel strategies have enabled marked progress in deciphering the membrane proteome over the last decade. In particular, the introduction of the FASP method for detergent removal, the establishment of SDC and its removal, and the application of Lys-C in the pre-digestion step are the most important methodical improvements, which have markedly increased the numbers of identified IMPs using the "classical strategy" that targets IMPs as whole molecules. The novel "divide and conquer" strategies oriented toward soluble glycosylated peptides (CSC, SPEG and glycol-FASP) or targeting hydrophobic segments using CNBr (hppK-CNBr and hpTC) have also significantly improved our knowledge of the membrane proteome. The most successful approaches in the current membrane proteome analyses addressed here are briefly summarized in Fig. 2. However, none of these workflows has the potential to completely decipher the membrane proteome in its vast complexity. Despite the high numbers of identified proteins, the classical trypsin-based strategies will inevitably neglect proteins that offer an insufficient number of (reasonably short and soluble) tryptic peptides. Similarly, glycopeptide-oriented analyses provide only a limited glyco-centric view of the membrane proteome, omitting non-glycosylated proteins. The analyses targeting the hydrophobic transmembrane alpha-helices also provide only an incomplete view of the membrane realm. However, the information provided by these three main approaches are complementary, and the combined forces of all three will probably be needed to obtain more comprehensive insights into the black box of the membrane proteome. For instance, a combination of glyco-capture with complementary analysis of the hydrophobic segments in one biological sample can be envisioned. If combined with SILAC labeling, such a combined analysis should provide a thorough and more complete snapshot. If further complemented by a classical detergent-(Lys-C)-trypsin strategy, such a "3-D" analysis would certainly provide unprecedented coverage of the membrane proteome.

Transparency document

The Transparency document associated with this article can be found in online version.

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