REVIEW

Technologies for plasma membrane proteomics

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Cell–cell and intracellular signaling are critical mechanisms by which an organism can respond quickly and appropriately to internal or environmental stimuli. Transmission of the stimulus to effector proteins must be coordinated, rapid and transient such that the response is not exaggerated and the overall balance of the cell or tissue is retained. Proteomics technology has traditionally been adept at analyzing effector proteins (such as cytoskeletal and heat shock proteins, and those involved in metabolic processes) in studies examining the effects of altered environmental or nutritional conditions, drugs, or genetic manipulation, since these proteins are often highly abundant, soluble and therefore amenable to analysis. Conversely, the proteins mediating the transmission of the signal have been generally under-represented, typically because of their low abundance. One mechanism that has overcome this to some extent is the advent of very high-resolution phosphoproteomics techniques, which have enabled temporal profiling of intracellular signal pathways *via* quantitative assessment of peptide phosphorylation sites. One group of proteins, however, that still remains under-represented in proteomics studies are those found in the plasma membrane (PM). Such proteins are crucial in sensing changes in the external environment and in stimulating the transmission of the signal intracellularly. This review examines PM proteins and appraises the proteomics approaches currently available for providing a comprehensive analysis of these crucial mediators of signal pathways. We discuss different strategies for enrichment and solubilization of these proteins and include discussion on cross-linking of PM complexes and glycoproteomics as the basis for purification prior to proteomic analyses.

Keywords:
Cell signaling / Enrichment / Glycoproteomics / MS / Plasma membrane proteomics / Solubilization

1 Introduction

1.1 Cellular signaling

Cellular signaling is the means by which organisms respond to external stimuli as well as keep strict control of the various processes occurring within and between cells. Signal pathways regulate complex processes, such as transcription and translation, proliferation, apoptosis, differentiation, metabolism and cell survival [1–4]. Altered or defective signaling pathways may result in abnormalities such as autoimmune reactions, uncontrolled proliferation or loss of apoptotic response resulting in pathogenesis of several diseases. As an example, type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by deficiency of insulin production believed to be a result of an autoimmune attack on the plasma membrane (PM) of the insulin-producing β-cells in the islets of Langerhans within the pancreas [4]. Substantial evidence has been collected to indicate that T1DM-associated β-cell death is caused by pro-inflammatory cytokines. Several of these cytokines are cytotoxic to β-cells, as they activate pro-apoptotic pathways [3, 5, 6] by cellular signaling across the PM. Ultimately, without clinical intervention through exogenous insulin, T1DM is lethal.

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Abbreviations: CNBr, cyanogen bromide; **EGF,** epidermal growth factor; **EGFR,** EGF receptor; **hMSC,** human mesenchymal stem cells; **IMP,** integral membrane proteins; **pIEF,** peptide isoelectric focusing; **PM,** plasma membrane; **RTK,** receptor tyrosine kinases; **SILAC,** stable isotope labeling of amino acids in cell culture; **T1DM,** type 1 diabetes mellitus; **TMR,** transmembrane-spanning region

Impaired cellular signaling often involves PM proteins and this is apparent in many cancers. For example, over-expression of the epidermal growth factor (EGF) receptor is observed in more than 50% of all carcinomas [7]. Ezrin is a protein that links F-actin to the cell membrane following phosphorylation and it has been associated with tumor progression and metastasis in several cancers including the pediatric solid tumors, osteosarcoma and rhabdomyosarcoma [8]. A recent study shows that over-expression of the intermediate-conductance calcium-activated potassium channel IK(Ca1) is likely to promote carcinogenesis in human prostate tissue [9]. In addition, NGEP-L, a gene encoding a PM protein, has been suggested to promote cell contact-dependent interactions of LNCaP prostate cancer cells [10]. Thus, the need to understand the role of PM proteins in cellular signaling, and within the dynamics of living cells, is evident.

1.2 PM proteins

PM proteins are critical in cell–cell communication and cellular signaling. PM proteins are, as the first barrier to the extracellular environment, involved in a multitude of processes enabling cells to sense and react to autocrine, endocrine and other environmental signals. They function as receptors for endogenous ligands, form channels or pores for low molecular weight compounds and other nutrients, and act as recognition or adhesion molecules [11]. Most PM proteins contain transmembrane domains with hydrophobic regions spanning the PM (transmembrane-spanning regions; TMR), and hydrophilic domains located on the external and cytosolic sides of the PM. Figure 1 shows the many different types of proteins embedded in or associated with the biological PM.

The PM itself is a phospholipid bilayer with fatty acids facing inwards and hydrophilic polar heads on the external and cytoplasmic faces. Within this structure are a number of proteins with different properties – (i) integral membrane proteins (IMPs) spanning the entire bilayer and with one or more TMR; (ii) IMP clustered in a multi-unit complex to form a pore or channel for the transport of nutrients and/or the removal of toxic compounds; (iii) IMP embedded in either the outer or inner surface of the lipid bilayer, but not spanning the entire membrane; and (iv) those proteins peripherally associated with the inner or outer face of the bilayer. Interestingly, many of these PM proteins are post-translationally modified by the attachment of complex *N*-linked glycans (glycosylation) – a property that can be exploited for the analysis of PM proteins in proteome projects.

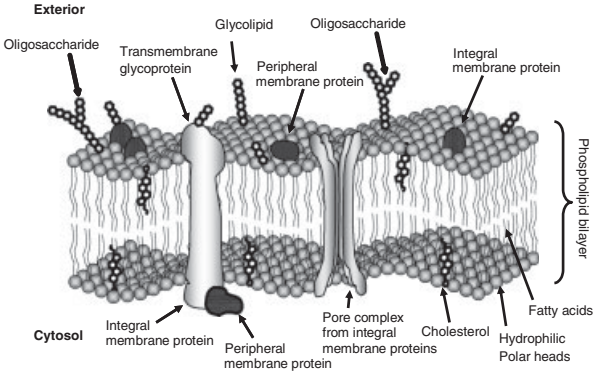

Figure 1. A schematic diagram of typical PM proteins in a biological membrane.

The protein domains exposed to the extracellular environment are often involved in cell–cell communication and binding of signaling molecules, whereas the domains located in the cytosol are important for anchoring cytoskeletal proteins and activation of intracellular signal pathways. Regulation of PM proteins is frequently achieved by reversible protein phosphorylation of distinct protein domains [12]. For example, mitogen stimulation often triggers a cellular response by activating entire phosphorylation cascades. These are initiated at a PM receptor and propagated into the cell by phosphorylation of adaptor, scaffolding or effector proteins. Because of their central role in cell signaling and transportation of molecules, PM proteins have been extensively targeted for drug design [13–17], emphasizing the enormous importance of these molecules.

The composition of the PM proteome and the abundance of certain PM proteins may be significantly altered during cell differentiation and disease progression. Consequently, PM proteins can be powerful biomarkers for disease development and prognosis, and may be useful clinical drug targets to prevent signal cascades associated with pathogenesis. Thus, the identification and characterization of PM proteins and their functions are critical for providing the molecular framework for understanding signaling and the effects of stimulation with various signal molecules. The latter is a crucial aspect in the discovery of new and better drugs and the development of strategies for treatment of diseases. This review will give an overview of the different strategies for isolation and characterization of PM proteins in large-scale proteomics projects, with an emphasis on novel developments likely to yield improved recovery of this important group of biomolecules.

1.3 PM proteins in signal transduction

PM proteins sit at the interface of the environment and the cellular response to that environment. Such proteins, or ''receptors'', are typically IMP with domain structures that allow the transmission of a stimulus from an external domain to an intracellular domain, *via* phosphorylation. The signal, once internalised, is then transmitted *via* a plethora of protein kinases and amplified such that multiple targets can be modified. PM receptors are generally classified into one of five groups: (i) G protein coupled receptor (GPCR)

family proteins; (ii) ligand-gated ion channels; (iii) receptor tyrosine kinases (RTK) [11]; (iv) integrins; and (v) Toll-like receptors. The best studied of these, at least from the proteomic perspective, are the RTK, where substantial work has been undertaken particularly in understanding signalling following EGF stimulation of the EGF receptor (EGFR) and the targets that transmit the signal downstream [18].

RTK are typically involved in detecting environmental signals such as growth factors or cytokines [19]. The EGFR is well characterized and it is known that stimulation by binding of the substrate leads to the formation of a dimer and tyrosine autophosphorylation within the intracellular domain occurring within seconds. This initial step is the catalyst for the alteration in EGFR binding, and signal initiators, such as phospholipase C-γ bind to the activated phospho-Tyr residues. Phospholipase C-γ catalyses the formation of second messengers such as inositol triphosphate and diacylglycerol that induce intracellular calcium-dependent signalling. Eventually, other phospho-Tyr-binding proteins (*e.g.* the Shc adaptor family of proteins) bind to activate EGFR, and are themselves phosphorylated. Thereby a cascade of events begins, leading to activation of the classical Ras/MAP kinase, and other, pathways [20]. Other well-described RTK include the insulin receptor, fibroblast growth factor receptors, and the platelet-derived growth factor receptors. The principles for their study by proteomic means has generally been to stimulate cells in culture with the growth factor of interest and to monitor signal pathways *via* purification of phosphopeptides and quantification by stable isotope labeling of amino acids in cell culture (SILAC) labelling [18, 21–23]. Phosphoproteomics has been reviewed extensively elsewhere; however, there is now an undoubtable need to use those approaches in conjunction with the best possible membrane enrichment strategies to determine signalling events occurring at the cell surface.

While proteomic studies of RTK-mediated signal transduction pathways have generally examined the intracellular phosphorylation profile following specific growth factor or cytokine stimulation, the actual effects at the PM receptor level have been largely neglected, most probably due to the technical challenges of membrane proteomics (see Section 2). Despite this there is increasing attention being paid to purification and separation of proteins from lipid rafts, which are PM micro-domains enriched in cholesterol and sphingolipids. These act as an organizational unit for vesicle trafficking and signalling [24, 25]. Others are attempting to purify membrane-related particles such as exosomes, micro-vesicles and micro-particles [26]. Such sub-cellular structures, enriched in PM proteins, including receptors, will be particularly useful for high-resolution studies of signal events.

2 Proteomics of PM proteins

The important role of the PM and PM proteins in pathogenesis has focused the research community on better means for providing a comprehensive analysis of such proteins in health and disease, or under altered environmental conditions or genetic background. The technologies encompassed under the term ''proteomics'' have often been employed to survey PM proteins. Several major studies have recently been published examining PM proteins from different cell culture models and/or tissues, including human embryonic stem cells [27], ovarian cancer cells [28], placental syncytiotrophoblasts [29], rat liver [30], B cells in mantle cell lymphoma [31], and renal cell carcinoma [32].

The study of PM proteins using high resolution and throughput proteomics remains challenging. While PM proteins are generally low in abundance, the major obstacle in their analysis is poor solubility. IMP, in particular, contain both hydrophobic and hydrophilic domains, which makes them difficult to purify and characterize on a proteomic scale. The vast majority of reports examining PM protein-enriched fractions are demonstrably lacking in information regarding IMP. In order to undertake a comprehensive analysis of mammalian PM proteins, several areas need to be considered. The analysis can be divided into three main experimental steps: (i) enrichment and purification of PM; (ii) solubilization of PM proteins; and (iii) separation, identification and characterization (analytical techniques). Each of these three steps provides experimental challenges and influences the success of the experimental design and the interpretation of the results.

2.1 PM and PM protein enrichment and purification

The most critical component of the experimental approach is the enrichment and purification of PM and PM proteins, with the major challenge being the presence of higher abundance, contaminating cytoplasmic proteins in the final protein extract prior to solubilization and analysis. High stringency and purity PM fractions are also difficult to obtain from samples where little material exists to begin with, for example, most tissue biopsies and those derived from laser capture microdissection [33]. Typically, this means PM studies rely on cell culture model systems, however, often only PM proteins of highest abundance are identified, many with poor reproducibility due to the presence of contaminating proteins from other organelles.

2.1.1 Precipitation and density gradient centrifugation

Sub-fractionation (or sub-proteomics) is a means for reducing the complexity and improving the dynamic range of an initially complex sample and thereby enhancing the identification of low abundance proteins or those specific to a research problem of interest [34–41]. The traditional approach to PM purification involves chemical precipitation and/or density gradient ultra-centrifugation. One such

method involves aqueous two-phase purification, where membrane (predominantly PM in eukaryotic cells) and membrane proteins are separated from soluble proteins according to hydrophobicity using, for example, dextran T-500 or PEG 3350 [42–45]. Recently, aqueous two-phase purification has been applied to the pre-fractionation of membrane proteins from rat brain and liver prior to MS analysis, leading to the identification of 42 and 67% IMP, respectively [42, 44]. PM can be specifically enriched by differential centrifugation or density sedimentation of whole cell lysates, tissue lysates or microsomes, where the PMs are separated from other sub-cellular organelles due to their different densities [46–50]. Zhang *et al.* applied sucrose density gradient centrifugation for the isolation of PM fractions from mouse liver, identifying 88 (50%) IMP from a total of 175 proteins [50]. Additional washing steps using high salt and high pH conditions can also improve cytosolic protein removal, particularly those loosely bound to the PM or trapped in vesicles formed after disruption [51]. Recently, Foster *et al.* combined sucrose gradient centrifugation with sodium carbonate extraction to enrich for PM proteins in human mesenchymal stem cells (hMSCs). They identified a total of 463 proteins, of which 122 (26.3%) were predicted IMPs [48]. However, even these successful studies confirm that PM fractions enriched by aqueous two-phase purification, conventional differential centrifugation or density sedimentation methods have typically been contaminated with significant levels of cellular organelles, making it difficult to compare protein expression profiles between two preparations [52, 53].

Strong non-ionic detergents, such as Triton X-114, can also be used for the separation of extracted hydrophobic and hydrophilic proteins. At 0°C the solution is homogeneous, however, when heated to above 20°C, the solution separates into an aqueous and detergent phase. The separation of the two phases becomes even more apparent at increasing temperatures and is affected by the presence of other surfactants [54]. Hydrophilic proteins separate to the aqueous phase, while hydrophobic IMPs are found in the detergent phase [54]. This method is highly efficient and has previously been used for the separation of glycosylphosphatidylinositol (GPI)-anchored proteins [55].

In a second study analogous to [24], the efficiency and reproducibility of combining sucrose centrifugation with sodium carbonate extraction for the enrichment of PM proteins was tested in preparation for phosphoproteomic studies of hMSC PMs [56]. PM proteins were extracted in the presence or absence of different phosphatase inhibitors (sodium pervanadate, calyculin A, a combination of two phosphatase inhibitor cocktails or no phosphatase inhibitors) and then phopshopeptides were compared by titanium dioxide and MS/MS. The independent sample preparations were highly reproducible with gene ontology classification of the cellular origin of the identified phosphoproteins from the four different experiments showing between 70.9 and 79.0% of the proteins were membrane proteins, with between 33.3 and 33.9% known to belong to the IMP class (Fig. 2). This study highlights a second very important issue; at this point, most phosphoproteomics studies of signal transduction following biological stimulation have neglected to specifically examine PM fractions. Signal pathways, however, must be stimulated *via* external sensor proteins and only specific examination of PM will enable determination of which sensor proteins are regulated. Overall, however, while the density gradient ultracentrifugation methods have long been utilized in many biochemical studies, it seems likely that they do not have the resolution to provide very highly purified PM fractions, and therefore, can only be used as enrichment procedures.

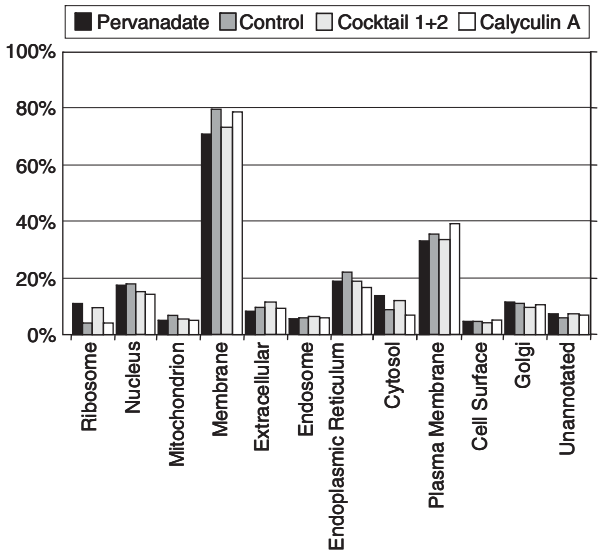

Figure 2. The efficiency and reproducibility of combining sucrose centrifugation with sodium carbonate extraction for plasma membrane protein enrichment. The efficiency and reproducibility of combining sucrose centrifugation with sodium carbonate extraction for the enrichment of PM proteins was tested in preparation for phosphoproteomic studies of hMSC PMs. Annotated information on the cellular location of the proteins identified was performed using ProteinCenter. Comparison of the cellular location of the proteins identified from the four experimental conditions (control cells *versus* cells treated with phosphatase inhibitor cocktails, sodium pervanadate or Calyculin A) are shown in this figure. Adapted from Thingholm [43].

2.1.2 Cross-linking PM protein complexes

A second strategy for purifying exposed IMP is to utilize protein cross-linking reagents to maintain membrane protein complexes in their close-to-native state. Once the cells are lysed, non-complexed proteins can be removed by size-exclusion chromatography, immunoprecipitation using antibodies against a membrane protein of particular interest, or by the use of a cross-linker that enables affinity purification. This method has been utilized by several

groups, mainly to understand surface protein topology within protein complexes [57]. An interesting variation of this approach was introduced by Freed *et al.* [58], who developed a method to allow biotinylation and surface protein cross-linking, thus providing a robust means of affinity purifying the resulting complexes. This method was applied to cell surfaces for the study of signal transduction. Very recent studies have improved the purification strategies by performing photocross-linking of membrane proteins with phospholipids [59]. In this study, mitochondrial membrane proteins were identified by cross-linking to phosphatidylcholine, leading to the identification of several novel constituents of this sub-sub-cellular fraction, and indicating that it may be a very effective approach for improving the identification of *bona fide* membrane proteins.

2.1.3 Cell ''shaving''

For a substantial time, researchers in proteomics have discussed the concept of cell-surface shaving for improving identification and relative quantification of surface-exposed membrane proteins. The concept revolves around the use of a protease in free solution around intact cells. Surface-exposed peptides are cleaved by the protease into the surrounding solution, collected and then analyzed by MS/MS for their identification (Fig. 3). The method, in theory, offers many advantages – first, it provides information about the surface topology of the cell (for example, which epitopes are exposed on the surface and capable of interacting with other surface molecules, nutrients, foreign particles, *etc.*); and second, success with such an approach would remove the need for solubilization of highly hydrophobic proteins, since surface-exposed peptides are generally soluble even from IMP that are insoluble overall.

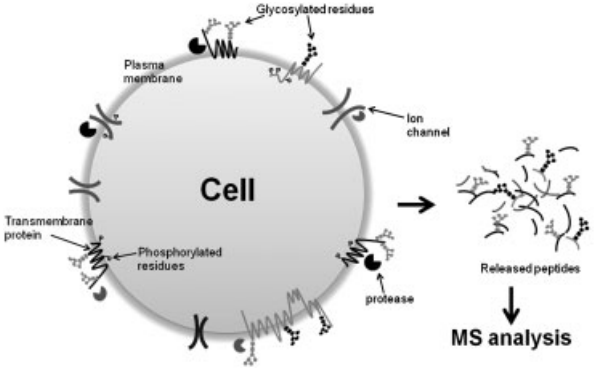

Figure 3. Cell ''shaving''. Surface-exposed peptides are released by protease digestion into the surrounding solution. Post-translational modifications present on the external domains of the plasma membrane proteins will still be intact on the released peptides. The peptides are then collected and analyzed by MS/MS for their identification.

Success, however, has been difficult to come by, mainly due to the instability of cells during the protease treatment and subsequent cell removal by centrifugation. Cell lysis, of course, results in significant contamination by abundant cytoplasmic proteins. Some success has been noted for bacterial systems (mainly the sturdy cell wall-containing Gram positives [60, 61]); however, significant numbers of cytoplasmic proteins remain in the identified protein lists, reducing confidence in the assignment of surface-exposed epitopes. Recent work using proteinase-K treatment on HeLa cells at high pH combined with microLC-MS/MS performed at higher temperatures, significantly improved the identification of IMPs [62] with 87% of the identified proteins predicted to contain at least three TMRs. In a further modification of the strategy [63], the term ''shave and conquer'' was introduced to describe phospholipase D treatment to remove GPI-anchored proteins from the cell surfaces of human and plant cells. While some successes have been noted, and the approach is undoubtedly of keen interest to many researchers, the problems associated with cell lysis are yet to be fully understood or overcome.

2.1.4 Biotinylation and immunoprecipitation

Another strategy for isolation of PM proteins is *via* the use of affinity enrichment, where cell surface membrane proteins are biotinylated on amino acid residues located in exterior domains and subsequently enriched using magnetic streptavidin beads [53, 64, 65]. In 2004, Zhao *et al.* utilized biotin/streptavidin to enrich PM protein fractions that were subsequently extensively washed using high concentrations of salts, or high pH, to remove any loosely membrane-associated, cytosolic proteins. This study identified 898 unique proteins from a human lung cancer cell line, of which 781 were reported to be localized to the PM and 67.3% of the annotated proteins were classified as IMPs [65]. This strategy, however, is limited by relatively low yields and is only applicable to cells grown in culture, and not tissue samples [16, 36].

Alternatively, PM proteins can be enriched using silica [66–68] or magnetic beads coated with specific antibodies [16, 36, 52], a method that can be applied to both cells grown in culture or tissue samples. Enrichment using immobilized monoclonal antibodies against known PM proteins is a specific and efficient technique for the additional purification of previously enriched PM fractions. Several groups have demonstrated success using this approach. Chang *et al.* used an antibody against the PM marker CD15 attached to super-paramagnetic beads for the purification of PMs from human neutrophils [52]. In 2006, Lawson *et al.* used the same method for the enrichment of PMs from rat liver and two different hepatic carcinoma cell lines [36]. Zhang *et al.* compared sucrose density gradient centrifugation with an optimized immunoaffinity protocol using secondary antibody super-paramagnetic beads for the enrichment of PM

proteins [16]. Mouse liver PM proteins were isolated by either of the two methods in the presence or absence of 0.1% BSA and the enriched proteins were subsequently separated by SDS-PAGE and analyzed by MALDI TOF-TOF MS/MS. Compared with sucrose density gradient centrifugation, the optimized immunoaffinity method gave a threefold increase in the number of identified PM proteins, and contamination with abundant, mitochondria-specific proteins was decreased twofold. A total of 248 unique proteins were identified, of which 67% were PM associated. An interesting variation of this method utilized cells coated with magnetic beads conjugated with antibodies against a cell type-specific cell surface molecule. After immobilizing the cells to the beads, cells were disrupted by nitrogen cavitation [69] and proteins bound to the beads were isolated by magnetic separation followed by density gradient ultracentrifugation. The authors stated a remarkable ~98% purity was achieved in their isolation of PM sheets [70].

2.1.5 Glycoproteomics as the basis for purifying PM proteins

A final approach that has captured attention as a means for conducting PM proteomics within cell culture and tissues is the analysis of *N*-linked glycoproteins. The addition of often complex glycans to proteins *via* asparagine (N) is close to ubiquitous on proteins localized to the PM. While glycosylation and composition of the glycans are undeniably of great interest in biomedical research, methods based on the analysis of *N*-linked glycoproteins are, at the very least, highly suitable for the enrichment of PM proteins. The traditional method for glycoprotein purification involves lectin affinity. Commonly used lectins include Con A (specific for glucose and mannose), soybean agglutinin (galactose and *N*-acetylgalactosamine) and wheat germ agglutinin (*N*-acetylglucosamine). Lectin-purified proteins are generally then separated by SDS-PAGE coupled to LC-MS/MS [71], 2-DE, or digested with trypsin and subjected to shotgun MS techniques. The use of multiple lectins for affinity purification has also been described [72]. Con A was recently utilized to purify *N*-linked glycoproteins from colon carcinoma cells, with 65% of the resulting identifications confirmed as PM proteins [73]. Several others have utilized the approach to examine body fluid proteomes, including urine, serum or plasma [61]. In a diagnostic variation of lectin chemistry, lectin-coupled protein arrays have been combined with SELDI-TOF MS profiling of serum samples from lung cancer patients to determine differences in sialylation patterns [74].

In more recent times, *N*-linked glycoproteomics of PM proteins has been facilitated by the use of hydrazine chemistry for purification of modified tryptic glycopeptides [75]. Complex protein mixtures are oxidized by sodium periodate, which converts *cis-diols* on carbohydrate groups to aldehydes. These aldehydes are then coupled to hydrazide groups on a solid support, allowing non-glycosylated proteins to be removed by extensive washing. Proteins bound to hydrazide are digested with trypsin and non-glycosylated peptides removed by washing. The final step removes bound glycopeptides by treatment with protein *N*-glycosidase F (PNGase F), resulting in the diagnostic deamidation of the glyco-modified Asn residue. The glycan remains associated with the hydrazide. A very impressive recent study used hydrazide chemistry coupled to multiple, parallel proteolytic digests to create a comprehensive glycoproteome of human liver tissue with the identification of over 900 glycosylation sites [76]. The original procedure has also recently been modified for high-throughput performance by the introduction of hydrazide surface supramagnetic silica particles [77]. Several groups have used this chemistry to aid in the purification of PM proteins. Lee *et al.* [78] utilized a dual strategy involving both lectin purification and hydrazide chemistry to identify 424 PM proteins from rat liver, while a similar approach was also used to examine HeLa cells [79]. Enrichment of glycopeptides by hydrophilic interaction chromatography has also been described [80], however, studies have concentrated on body fluids, including human breast milk [81], serum [82] and plasma [83]. Another alternative is the concept of the ''sialiome'' (the profile of glycoproteins containing glycans with terminal sialic acids) [84]. The technique relies on the use of TiO_2 chromatography, previously used for purifying phosphopeptides from complex mixtures [56, 85–95]. In summary, the rapidly evolving field of glycoproteomics will most likely generate significant improvements for identifying PM proteins, independently of a research interest in their glycosylation status.

2.2 Separation of complex PM protein fractions

Following successful enrichment/purification of PM proteins, the resulting samples remain complex and further fractionation is needed to increase the eventual protein/peptide identification coverage. Fractionation methods are typically focused on either separation at the protein or peptide level. Several methods are available for protein separation including, (i) ''slice and dice'' SDS-PAGE coupled to LC/MS/MS; and (ii) 2-DE; as well as methods for peptide separation post-proteolytic digest, including (iii) peptide isoelectric focusing (pIEF); and (iv) multidimensional peptide LC coupled to MS/MS.

2.2.1 2-DE and SDS-PAGE/LC-MS/MS of PM proteins

For many years, a very popular fractionation method has been SDS-PAGE [96–98], either as a sole separation technique, or in conjunction with IEF for 2-DE. At the protein level, both techniques have major limitations that reduce

their utility for fractionation of PM proteins. 2-DE gels, in particular, poorly resolve basic or hydrophobic proteins and those with >3 TMR [99]. Another major disadvantage of both methods is limited sensitivity and thus the difficulty in detecting lower abundance proteins, as well as the narrow dynamic range, meaning these limitations have lead to the use of peptide-focused, liquid-based strategies [100]. A variation on the gel-based technique that has shown some benefits for PM protein fractionation is the use of SDS-PAGE ''slice and dice''. In this approach, complex mixtures are separated by SDS-PAGE and the resulting gel ''sliced'' into equal bands throughout the full lane. Each band is then ''diced'' into smaller pieces and subjected to a proteolytic digest to release peptides that are then identified by RP LC-MS/MS. This method has the advantage of utilizing SDS for efficient solubilization of hydrophobic proteins, yet there remain difficulties for comparative quantitation between biological samples.

2.2.2 pIEF

Recently, a technique previously used only for gel-based protein separation, IEF, has enabled both protein and peptide fractionation to be performed in solution, and new protocols have been developed using various detergents, alternative denaturants and thiol oxidation agents [101, 102] to improve focusing [99]. pIEF in IPG strips [103], free-flow electrophoresis, or in liquid isoelectric focusing [104] has the potential to provide enhanced sensitivity, as well as improved reproducibility for peptide separations. A recent study used IPG pIEF to identify 626 membrane proteins from rat liver, although this represented only 42% of the identified proteins [105], suggesting that deeper mining into enriched membrane proteomes may also increase the identification rate of proteins contaminating from other subcellular fractions. Free-flow electrophoresis is an approach suitable for separation of organelles [106, 107], proteins and peptides and is highly reproducible, providing exquisite separation, with possible collection of up to 96 fractions [104]. CE is also a potential separation methodology [108, 109].

2.2.3 LC

By far the most common technique employed for sample fractionation in PM proteomics involves separation of proteins or, more routinely, peptides, by HPLC [110, 111]. As biological samples are highly complex, a multi-dimensional separation/fractionation approach is often required where several methods are combined [112–114]. Multidimensional protein identification technology (MudPIT) combines strong cation exchange chromatography, either on- or off-line, with RP chromatography [112], generally coupled directly with a mass spectrometer for MS/MS sequencing. Strong cation exchange has a high loading capacity and by combining it with the strong resolving power provided by RPLC, a highly efficient separation from within a complex mixture can be obtained [100, 115]. This 2-D LC strategy is now routinely employed for the analysis of PM proteins and IMP, since hydrophilic peptides from otherwise insoluble proteins are amenable to rapid analysis. Second, the commercial introduction of isobaric mass ''reporter'' tags (for example, ICAT [116, 117] or isobaric tags for relative and absolute quantitation [iTRAQ^{TM}]) [118], as well as SILAC [119, 120], has greatly facilitated the relative quantitation of proteins across multiple biological samples. The ability of iTRAQ and SILAC to label the vast majority of all tryptic peptides means that experimentally fewer technical replicates are needed for confident statistical analysis. Statistics and error calculations can be made from multiple parallel peptide quantifications generated from the same protein.

2.2.4 Phosphoproteomics

For studies where the focus is on cell signaling, enriched PM fractions may be directly digested and subjected to peptide-based phosphoproteomic strategies. The most commonly employed strategies involve peptide-focused analysis of enriched phosphopeptides, typically by IMAC [121–124] or TiO_2 micro-chromatography [125–128], each of which have altered binding affinities for phosphopeptides, and in fact may best be utilized together in a sequential fashion [129–131]. The field of phosphoproteomics has been extensively reviewed recently [132–134] and the different phosphoproteomic strategies will therefore not be focused upon here.

2.3 Solubilization of PM proteins

When PM fractions have been successfully enriched, one still faces the challenge of solubilizing PM proteins in order to perform further proteomic analysis. One technique involves repeated freezing and thawing, however, this step is only able to solubilize proteins loosely associated with the PM [34]. Highly hydrophobic IMP must be solubilized using various detergents [51, 135–137] following protein extraction, but depending on the downstream separation/fractionation technique. For example, the use of chaotropic reagents such as urea or guanidine hydrochloride, or strong ionic detergents such as SDS, may not be compatible with some separation techniques, or furthermore may inhibit the ability of proteases such as trypsin to function optimally [135, 138]. Most importantly, the known biochemistry regarding the system under investigation should be taken into account. It is often the case that methods for extracting PM proteins for enzyme assays, among other bioanalytical techniques, exist in the literature. As a case study, several

groups have examined liver tissue from the rat as a model for PM proteomics. For example, Lin and Fain combined sodium cholate and polidocanol to solubilize rat liver PMs for the study of the (Ca^{2+}–Mg^{2+})-ATPase [136], while Josic and Zeilinger used a combination of different solubilization agents and Triton X-100 [135]. In a further study, Josic *et al.* used both of the previously described approaches [34]. After solubilization of PM, they precipitated the detergent-resistant proteins using ethanol/acetone to remove lipids. The proteins were subsequently solublized using 8 M urea containing the zwitterionic detergent CHAPS [34]. This, however, does not solubilize membrane proteins containing many TMR regions, or those with a high GRAVY or solubility score constant [139]. Clifton *et al.* used EGTA (pH 7.4) containing octyl-glucopyranoside for the extraction of detergent-resistant proteins prior to SDS-PAGE and LC-MS/MS in a study of rat liver PM proteins [140]. In a subsequent study, the same group utilized a sequential extraction procedure using repeated freeze/thawing as a first step, followed by washes with different salt solutions and/or high pH (pH 11). As a third step, IMPs were solubilized using different detergents and finally, detergent-insoluble proteins were extracted by calcium chelation with EDTA or EGTA in combination with a detergent such as octylglucoside or CHAPS [34].

As mentioned above, PM proteomics faces additional challenges, since many suitable detergents and chaotropes for PM protein solubilization interfere with downstream separation, particularly LC. Furthermore, these chemicals may also introduce noise into analytical techniques such as ESI MS and therefore must be removed prior to analysis [141]. Several methods exist to achieve this, however, most result in loss of analyte and are therefore not necessarily compatible with studies examining low yield samples. Detergents are most typically removed by precipitating the proteins using trichloroacetic acid (TCA), a combination of chloroform and methanol, or simply organic solvents such as acetone. Beyond the overall loss of protein from the sample following precipitation, it is also possible that very hydrophobic proteins, in particular, may be lost at this step [34, 36]. To avoid this, Blonder *et al.* combined carbonate extraction and solubilization using surfactant-free organic solvent followed by tryptic digestion prior to MS analysis for the study of PM proteins in *Deinococcus radiodurans* strain R1 cells [141]. Often, detergents can be removed by precipitating the proteins using TCA, the combination of chloroform and methanol, or simply organic solvents such as acetone. However, very hydrophobic proteins may be lost at this step [34, 36].

In a phosphoproteomic study of hMSC, enriched PM fractions were solubilized using 2 M thiourea and 6 M urea, a strategy previously employed successfully in conjunction with 2-DE proteomics studies. In a modification of this strategy, the PM fractions were freeze-thawed at −80°C in this buffer, since urea forms sharp crystals upon freezing that can roughly break the sample and thereby improve solubilization. Furthermore, thiourea has been shown to specifically improve solubilization of membrane proteins [142]. A summary of the main methods for membrane protein enrichment and solubilization is listed in Tables 1 and 2.

3 Proteomic analytical strategies for the analysis of PM proteins

3.1 Choice of analytical approach

There are two main strategies for MS analysis of proteins, commonly referred to as ''top-down'' or ''bottom-up'' MS. In ''top-down'' MS, intact proteins are analyzed by MS/MS, often following multi-dimensional separation using, *e.g.* gel electrophoresis to separate the proteins [143]. The ''top-down'' approach has the benefit of giving closer to full sequence coverage, as well as providing valuable information on protein structure and post-translational modification [144]. This approach, however, requires high-resolution instrumentation, including FT-ICR MS, electron capture dissociation or electron transfer dissociation capabilities; however, recent reports suggest that even quadrupole TOF instrumentation can be modified to successfully undertake top-down approaches [145]. At this time, top-down MS has been particularly successful in identifying post-translational modifications in individual proteins, including histones [146] and myosin binding protein C [147], as well as sequence polymorphisms [148]. Top-down MS holds substantial promise for fully delineating IMP, due to the development of IMP-specific LC protocols [144] and the soft-ionization techniques listed in Section 2. For example, Zabrouskov and Whitelegge [149] showed improved coverage within two TMRs for the 8 kDa ATP synthase c-subunit.

3.1.1 Proteases suitable for PM protein digestion in conjunction with ''bottom-up'' MS

The vast majority of proteomics studies are, however, performed using the ''bottom-up'' strategy (described in Section 2.2.3). In this workflow, intact proteins are cleaved, most commonly using proteases such as Asp-N, Glu-C, Lys-C, trypsin or less specific enzymes including pepsin and proteinase-K (Table 3). Trypsin is the typical choice, and this is not without reason as it is highly specific, cleaving on the *C*-terminal side of lysine and arginine residues except when a proline residue is positioned directly on the *C*-terminal side of the cleavage site. Trypsin digestion for most proteins creates a series of peptide in the mass range compatible with nearly all MS instruments. In addition, trypsin is a small enzyme, which facilitates ''in-gel digestions''. While trypsin is the protease of choice for most protemics analyses, often other enzymes are

Table 1. A summary of the main methods for membrane enrichment

Membrane enrichment strategy	Principle	Origin of sample	Results	Ref.
Two-phase purification	Separation of membrane proteins from soluble proteins according to hydrophobicity	Rat liver	67% were integral membrane proteins	[29]
Differential centrifugation or density sedimentation	Separation of membrane proteins from other sub-cellular organelles according to densities	Mouse liver	50% were integral membrane proteins	[37]
Wash with high salt or high pH conditions	Removing cytosolic proteins loosely bound to the PMs or trapped in vesicles	Rat liver	Efficient removal of peripheral membrane proteins	[38]
Combination of sucrose centrifugation and sodium carbonate extraction	Purification of membrane proteins and subsequent removal of cytosolic proteins loosely bound to the plasma membranes	hMSC	57.3% were integral membrane proteins	[43]
Biotinylation enrichment	Enrichment of biotinylated plasma membrane proteins using streptavidin beads	Human lung cancer cell line	67.3% were integral membrane proteins	[52]
Enrichment of *N*-linked glycoproteins	Triton X-114 phase partitioning, lectin affinity and hydrazide chemistry for enrichment followed by PNGase F release	Rat liver	Lectin affinity: 65% were membrane proteins; Hydrazide chemistry: 78% classified as membrane proteins	[65]
Enrichment using a solution of cationic colloidal silica particles	Enrichment by electrostatic cross-linking to silica particles	Rat lung microvascular endothelial cells	81% were classified as plasma membrane proteins	[54]
Enrichment using secondary antibody super-paramagnetic beads	Enrichment using beads coated with antibodies against known plasma membrane proteins	Mouse liver	67% were plasma membrane or plasma membrane associated proteins	[13]

Each method/strategy is listed with details of the principle, an example of an application, the origin of sample for the application as well as a reference. The references in the table refer to the application studies, which are not necessarily the original references to the particular methods.

also useful either in parallel (a combination of enzymes can increase overall proteome coverage [76], or as an alternative. For example, glycopeptide analysis may be facilitated by the use of proteinase-K following trypsin digestion to remove interfering non-glycosylated peptides [150]. For the specific analysis of PM proteins, some studies have employed Lys-C as an initial step in the proteolytic procedure. Lys-C cleaves on the *C*-terminal side of lysine residues, and hence does not interfere with the subsequent trypsin digestion, but more importantly is highly efficient in high concentrations of urea. Urea is a protein denaturant; effectively disrupting non-covalent bonds in proteins as well as the hydrophobic interactions in the PM. Thereby urea makes Lys-C recognition sites in proteins readily available for cleavage and Lys-C pre-digestion improves the subsequent tryptic digestion, which is performed after dilution of urea [56].

Proteins can also be cleaved using enzyme-free approaches or by combinations of non-enzymatic and enzymatic hydrolysis. At the beginning of the 20th century, isolation of mono-amino acids was performed using aqueous hydrochloric acid as the hydrolyzing agent, and subsequently a saturated alcoholic solution of hydrogen chloride for esterification [151]. In 1913, Weizmann and Agashe optimized this protocol using only a saturated alcohol solution of hydrogen chloride to serve both as a hydrolysing and an esterifying agent. It was found that the solution only resulted in partial protein cleavage [151]. Partial acid hydrolysis has been used for the generation of peptides from proteins separated by SDS-PAGE for peptide mapping and Edman sequencing [152, 153]. In 2001, Li *et al.* introduced the use of formic acid for the chemical cleavage at aspartyl residues for protein identification [154]. These techniques are no longer typically employed due to the better specificity provided by enzymatic digestion, although some studies have utilized cyanogen bromide (CNBr) cleavage to examine unique sequences within individual proteins [155], or to improve identification of the *C*-termini of proteins [156]. CNBr has been applied in several PM protein studies [157–160]. In 1998, Ball *et al.* used CNBr to chemically cleave IMPs prior to MS analysis in a study to map bacteriorhodopsins and rhodopsin [158]. The enriched and precipitated protein was resuspended in 50–70% TFA and cleaved using approxi-

Table 2. A summary of the main methods for membrane solubilization

Membrane solubilization strategy	Principle	Origin of sample	Results	Ref.
Repeated freezing and thawing	To solubilize proteins loosely attached to the plasma membrane	Rat liver and hepatocellular carcinoma Morris hepatoma 7777	Solubilization gave reproducible and reliable results in a comparative proteomics study	[21]
Different detergents followed by protein extraction using chaotropic reagents or strong ionic detergent	EGTA, pH 7.4 containing octyl-glycopyranoside for the extraction of detergent-resistant proteins	Rat liver	Identification of members of the annexin family in the detergent-insoluble fraction of rat Morris hepatoma plasma membranes	[127]
Enrichment by phase separation of extracted hydrophobic and hydrophilic proteins using detergent	At zero degrees the detergent Triton X-114 is homogenous, but at 20 degrees it forms a detergent phase containing membrane proteins, and an aqueous phase containing hydrophilic proteins	Method setup testing the efficiency of enriching hydrophilic and hydrophobic proteins in two different phases.	Hydrophilic proteins (serum albumin, catalase, ovalbumin, *etc.*) were solely identified in aqueous phase, whereas hydrophobic membrane proteins (acetylcholinesterase, bacteriorhodopsin, and cytochrome *c* oxidase) were identified in detergent phase.	[41]
Carbonate extraction and solubilization using surfactant-free organic solvent	The use of surfactant-free organic solvents to solubilize membrane proteins to avoid interference with subsequent MS analysis	PM proteins in *D. radiodurans* strain R1 cells	An extensive coverage of the *D. radiodurans* membrane sub-proteome	[128]

Each method/strategy is listed with details of the principle, an example of an application, the origin of sample for the application as well as a reference. The references in the table refer to the application studies, which are not necessarily the original references to the particular methods.

Table 3. A summary of the most commonly used strategies for protein digestion in ''bottom-up'' studies

Enzyme	Cleave *C*-term. to	Cleave *N*-term. to	Do not cleave
Trypsin	Lysine and arginine		Proline
Chymotrypsin	Phenolalanine, tyrosine, tryptophan, leucine, isoleucine, valine and methionine		Proline
Lys-C	Lysine		Proline
Glu-C	Glutamine		
Asp-N		Aspartic acid	
Arg-C	Arginine		Proline
CNBr	Methionine		
Formic acid	Aspartic acid		
Proteinase-K	Non-specific	Non-specific	

The enzymes and chemicals are listed along with their amino acid specificities. In addition, details on whether the enzymes cleave when a proline residue is positioned next to the cleavage site are shown.

mately 2 mg CNBr/mg membrane protein. The solution was incubated in a dark oxygen-free environment for 18 h at room temperature after which the resulting peptides were separated by RP-HPLC and analyzed using ESI-MS or MALDI-TOF MS. This method achieved almost 100% sequence coverage of the IMP rhodopsin [158]. A second strategy was employed by Washburn *et al.* using formic acid to partially solubilize membranes from insoluble yeast cell fractions [157]. This study used CNBr to cleave IMP segments from the disrupted membranes, and then digested these fragments further with trypsin. They identified a total of 1484 proteins of which 31 were categorized as membrane proteins having three or more predicted TMR [157].

The digestion procedure itself may be optimized in several ways. In 1986, the use of microwave-assisted organic

synthesis was demonstrated [161, 162]. Today, microwave irradiation is used to assist in a number of chemical or enzymatic reactions, such as the hydrolysis of proteins and peptides [163–168]. Recently, Zhong *et al.* presented the use of microwave-assisted acid hydrolysis for rapid protein digestion prior to MS/MS protein identification [169]. They used a 25% TFA aqueous solution to dissolve proteins, followed by microwave irradiation for 10 min. This detergent-free method generated peptide mixtures that could be directly analyzed by LC-MALDI MS without the need for extensive sample cleanup. Microwave irradiation has also been implemented to improve enzymatic digestion using trypsin.

4 Concluding remarks

PM proteomics is central to our understanding of how cells work. They are the interface between a cell and its environment, allowing nutrients to pass into the cell and waste products to be removed. They interact directly with other cells, both self and foreign, and therefore play an active role in immunity. Both of these functions are linked by cell signaling. Changes in the micro-environment surrounding a cell, due to nutrient limitation, stress, or foreign particles must be sensed by surface-exposed proteins that are responsible for transmitting these signals intracellularly, such that signal pathways may be activated and gene regulation altered. Despite this, proteomics of the PM remains technically challenging; however, significant improvements have been made from membrane purification, through to membrane protein separation, and even in the analytical steps needed to provide full coverage of these proteins. It is to be anticipated that with these technical breakthroughs, and with more likely in the future, that PM proteomics will be an enabling approach for the discovery of new biological pathways and functions, as well as for the design of better therapies and intervention strategies.

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