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Coupling enrichment methods with proteomics for understanding and treating disease

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Abstract

Owing to recent advances in proteomics analytical methods and bioinformatics capabilities there is a growing trend toward using these capabilities for the development of drugs to treat human disease, including target and drug evaluation, understanding mechanisms of drug action, and biomarker discovery. Currently, the genetic sequences of many major organisms are available, which have helped greatly in characterizing proteomes in model animal systems and humans. Through proteomics, global profiles of different disease states can be characterized (e.g. changes in types and relative levels as well as changes in PTMs such as glycosylation or phosphorylation). Although intracellular proteomics can provide a broad overview of physiology of cells and tissues, it has been difficult to quantify the low abundance proteins which can be important for understanding the diseased states and treatment progression. For this reason, there is increasing interest in coupling comparative proteomics methods with subcellular fractionation and enrichment techniques for membranes, nucleus, phosphoproteome, glycoproteome as well as low abundance serum proteins. In this review, we will provide examples of where the utilization of different proteomics-coupled enrichment techniques has aided target and biomarker discovery, understanding the drug targeting mechanism, and mAb discovery. Taken together, these improvements will help to provide a better understanding of the pathophysiology of various diseases including cancer, autoimmunity, inflammation, cardiovascular disease, and neurological conditions, and in the design and development of better medicines for treating these afflictions.

Keywords

Biomarker discovery; Drug target discovery; Exosomes; Membrane proteomics; mAb discovery

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1 Introduction

With enhancements in proteomics techniques, there has been a surge in world-wide efforts aimed at large-scale protein analysis of biological samples. A global human proteome project is now underway to facilitate understanding of the relationship between physiological changes in organisms and protein abundance, subcellular localization, and functions in different tissues and environments [1]. Indeed, a large number of biomarkers for different diseases including cancer have been discovered in the past two decades using MS-based proteomics approaches [2, 3]. Proteomics has proven to be useful in cancer research because the complexity of tumorogenesis, cancer progression, tumor relapse, and metastasis may involve complex protein networks. The recent application of proteomics in cancer-related research is evident by the steadily increasing number of publications from 2000 to 2010 followed by sustained numbers through 2013, as shown in Fig. 1 (http://www.ncbi.nlm.nih.gov/pubmed). The acceleration of the use of genomics and transcriptomics in cancer research has been due to advancements in the core technologies used to sequence and analyze nucleic acid. As seen from Fig. 1, proteomics, while lower than genomics and transcriptomics, is still far exceeding other types of omics technologies, such as metabolomics, lipidomics, and glycomics. However, the recent advancements in MS technologies will hopefully accelerate the use of all of these areas in the coming years. Among the omics research community, integration of proteomics and other omics technologies such as RNA sequencing (RNA-seq) is desirable [4]. There is an ongoing effort on the integrative analysis of data types from multiple omics sources such as The Cancer Genome Atlas Project (TCGA) that integrates endometrial carcinoma data characterized by sources such as mRNA, protein, DNA methylation, and so on [5]. With increasing usage of multiple omics techniques, there is also a need for development of user-friendly tools capable of performing analysis of data from multiple omics sources [4].

The number of publications has increased over the past 10 years in part because of the need in many areas as illustrated in Fig. 2. MS-based proteomics techniques involving different ionization methods such as EI (electron ionization), ESI, and MALDI with quantification power can now be used to interpret the mechanism of action of various drugs, elucidate novel biomarkers, identify new targets, and provide antigen-specific antibody isolation. Within proteomics research, there is an increasing focus on changes in specific groups of proteins which may be present at relatively low levels. For example, secreted proteins, also referred to as the secretome, can play important roles in cell–cell communication, growth, and, as they can reflect the various stages of pathophysiological conditions [6], represent a useful source of biomarkers and potential targets to treat disease. Various proteomics approaches have been used to discover biomarkers in blood/plasma, body fluids, and tissues [7,8], exosomes [9], and conditioned media from cultured or primary cells [10].

In addition to the secretome, post-translationally modified proteins including glycosylated [11, 12] and phosphorylated ones represent another subclass of targets since they are involved in regulating biological processes. Dysregulation of kinase signaling pathways is commonly associated with various cancers [13] and gastrointestinal stromal tumors [14]. Phosphoproteomic approaches can also assist in identifying appropriate therapeutic targets as well as elucidating drug action on pathophysiological pathways. In addition to

MS [15], other high throughput proteomic technologies have been developed for studying phosphorylation including peptide arrays [16, 17], reverse-phase protein microarrays [18], and antibody arrays [19].

This review will explore the multiple identification and enrichment proteomics methods, as shown in Fig. 3, used for target discovery, biomarker discovery, and understanding the mechanism of action of drugs.

2 Target discovery

Comparative quantification of low abundance and complex proteins is becoming an attractive field since it can provide novel drug targets for therapeutic intervention and to meet these developments, multiple proteomic and associated technologies are being implemented. As shown in Fig. 3, the serum depletion method reduces the complexity of a sample by removing high abundance proteins, thereby enriching the low abundance proteins. Low abundance phosphopeptides can be enriched using titanium dioxide (TiO_2) incubation as a result of the high affinity of TiO_2 to negatively charged phosphopeptides. Similarly, hydrazide chemistry can be used to capture glycopeptides and cationic nanoparticles such as colloidal silica can provide the high level enrichment of low abundance membrane proteins.

In particular, development of an effective strategy for membrane protein analysis is highly relevant to diseases where cell surface receptor signaling plays a vital role. For example, cell surface proteins have attracted substantial interest from cancer researchers in areas ranging from molecular diagnosis to therapy.

However, comparison of membrane proteins from normal and diseased tissues using standard extraction methods that also isolate whole cell components makes this task difficult. For this reason, recent advances in coupling proteomics methods with membrane protein enrichment protocols [20–22] are especially useful for novel target discovery.

2.1 Membrane proteomics

Membrane proteins play important roles in numerous diseases and can be potential drug targets. Various methods, as discussed below and in Table 1, have recently been developed that could potentially overcome challenges in studying membrane proteomics.

2.1.1 Ultracentrifugation techniques for membrane proteome enrichment—

Membrane proteins can be enriched by the removal of cytosolic proteins under high pH conditions by using ultracentrifugation. For example, the membrane proteome of postmortem brain tissues from normal and Alzheimer’s disease cases were compared using two simultaneous ultracentrifugation procedures. The simultaneous centrifugation and treatment with high pH removed the loosely associated proteins to improve the membrane proteome quality [23].

In another application, proteome profiles of plasma membrane enriched microdomains (MD) involved in cell signaling, transport, and proliferation from human renal cell carcinoma (RCC) and adjacent normal kidney (ANK) were compared using gradient ultracentrifugation

[24]. Ninety-three proteins were identified in MD isolated from RCC and 98 proteins in MD isolated from ANK. Western blot analysis indicated differentially expressed proteins such as Thy1, VDAC, and DPEP, which may represent potential biomarkers of cancer progression [24]. In another study, the apical plasma membrane proteome of a multinuclear synctiotrophoblast was enriched using ultracentrifugation and sucrose gradient centrifugation in conjunction with 1D SDS-PAGE and ESI-MS [25]. Two hundred ninety-six nonredundant proteins were identified, of which about 60% were integral and peripheral membrane proteins [25].

2.1.2 Biotinylation coupled proteomics for cell surface proteome enrichment

—Biotinylation of whole cells can be used to enrich low-abundance cell surface protein isolation by taking advantage of the high affinity of avidin, streptavidin, or neutravidin for biotin. This method works by conjugation of multiple biotin molecules to target proteins of interest to form a biotin complex. Surface biotinylation with reactive chemical derivatives of biotin followed by purification on avidin beads allows selective cell surface proteome profiling. The method often involves resuspending whole cells in a solution of a biotin derivative such as sulfo-NHS-SS-biotin. After the capture of the biotin complex on streptavidin or avidin-based supports, the biotinylated proteins can be cleaved using reducing reagents [26].Combining cell-surface protein enrichment using biotinylation and iTRAQ technology has been used to detect differentially expressed proteins in endometrial cancer and healthy cells [27]. Out of 272 overexpressed proteins, overexpression of bone marrow stromal antigen 2 (BST2) on cancer cells was investigated further. Administering a monoclonal antibody targeting BST2 (anti-BST2) resulted in growth reduction of BST2-positive endometrial cancer cells in SCID mice identifying BST2 as a possible therapeutic target [27]. In this way, biotinylation coupled with MS profiling has facilitated the identification of critical, low-abundance membrane proteins [28].

2.1.3 Glycoproteome enrichment—

PTMs including glycosylation offer high diagnostic and therapeutic potential and thus glycoproteomics is another area for target identification [29–39]. However, there are many challenges to achieving efficient analysis of a complex glycoproteome containing wide concentration ranges in a complex biological environment due to the severe masking effects of highly abundant proteins and the alteration in the stoichiometric ratios of glycosylated forms of proteins. To overcome these obstacles, various enrichment strategies for glycoproteins have been developed [40]. As glycoproteins are localized on the cell surface or are secreted, *N*-glycoproteome enrichment methods facilitate identification and expression comparisons of membrane proteins. There are multiple glycoproteome enrichment methodologies, some of which are:

1. Gravity-flow columns involve use of a glycoprotein enrichment resin (such as a phenylboronic acid-based resin containing a ligand, attached to agarose beads, which binds to sugar residues of glycoproteins [41]) to capture the glycoproteins.
2. Cell surface capture involves treating glycoproteins of the plasma membrane by oxidizing cells with sodium-meta-periodate followed by covalent chemical labeling with biocytin hydrazide (BH), which is a biotin containing hydrazide.

The BH-labeled glycopeptides can then be analyzed by LC-MS/MS for peptide identification [42].

3. For enrichment of *N*-linked glycopeptides, SPE of *N*-linked glycopeptides (SPEG) is highly preferred for both identification and quantification [43].The SPEG method involves proteolysis of proteins into peptides followed by coupling oxidized glycosylated peptides to hydrazide beads and removing non-glycosylated peptides. The amino-termini of glycopeptides can be labeled with succinic anhydride or other reagents followed by peptide-*N*-glycosidase F (PNGase F) to release *N*-linked glycopeptides, which can be identified and quantified by MS [44].

A highly selective glycopeptide enrichment method combining several of these glycoproteomics methods including cell surface capture and hydrazide derivatization has been applied to isolate cell surface proteins [45]. This method has several advantages over other methods as it provides complete solubilization of membrane proteins, enriches glycopeptides instead of glycoproteins that eliminates potential steric hindrance during capture, ensures robustness and tolerance to stringent washes, decreases sample loss, and offers shorter sample processing times [45]. Processing steps include cell lysis, microsomal fraction, denaturation and digestion, glycopeptide capture, LC-MS, and bioinformatics analysis. The success of this method, particularly suited to in vitro cell cultures, relies on complete digestion of the samples [45].

In another study, a modified cell surface capture strategy involved oxidizing the oligosaccharides on cell surface proteins followed by cell lysis and coupling oxidized proteins onto a hydrazide resin followed by MS analysis for quantification of glycoproteins and other cell surface proteins [46]. This strategy was tested on two biological replicates of chang liver and HepG2 human cell lines. Out of 341 identified glycoproteins, 33 exhibited significant changes in expression between the two cell lines. Western blot was used to validate the higher expression of extracellular matrix metalloproteinase inducer (EMMPRIN) and basal cell adhesion molecule in HepG2 cells, both of which are associated with the malignant potential of tumor and liver cancer [46].

Recently, a detailed large-scale LC-MS/MS data set on the membrane proteome and *N*-glycoproteome of the BV-2 microglia line was reported [47]. This study employed multiple strategies including crude membrane fractionation, filter-aided sample preparation (FASP)-based differential sample preparation, and *N*-glyco-FASP-based glycopeptide enrichment. A total of 6928 unique protein groups and 1450 unique N-glycosites and 760 unique glycoproteins were identified [47]. Among the identified glycoproteins, receptors, transporters, peptidases, and ion-binding proteins were enriched [47].

Quantitative glycoproteomics based on hydrazide chemistry was used to study cell surface and serum proteins in human serum and a prostate cancer epithelial cell line [48]. This method was found to be useful in reducing serum sample complexity with detection of 2.5 peptides per protein on average. For the prostate cancer epithelial cell line, this method could identify proteins such as HSPG2 and SSRA that were not previously identified by a 2DE gel method [48].

A targeted proteomics approach involving galactose oxidase and aniline-catalyzed oxime ligation (GAL) and periodate oxidation and aniline-catalyzed oxime ligation (PAL) was used for chemical tagging and identification of glycosylation sites on proteins of cells with an altered sialylation status [49]. Immobilized streptavidin was used to pull down the PAL and GAL labeled and biotinylated glycoproteome and the glycopeptides were released with *N*-glycosidase treatment to be analyzed in LC-MS/MS [49]. One hundred and eight nonredundant glycoproteins were identified by combining both methods [49].

2.1.4 Enrichment of plasma membrane proteome with cationic nanoparticles

—Isolation and study of the plasma membrane proteome is especially challenging due to the low abundance and hydrophobicity of the proteins. In addition, a variety of post-translational and chemical modifications leads to a dynamic population of these proteins on the cell surface. Cationic nanoparticles synthesized from Fe_3O_4 (magnetite) coated with Al_2O_3 were used to enrich the low abundance plasma membrane proteome of human multiple myeloma RPMI 8226 cells for analysis of integral proteins and proteins from both the inner and outer surfaces [50]. Fe_3O_4 (magnetite) nanoparticles were synthesized and coated with Al_2O_3 and capture of membrane proteins was then compared to commercial aluminosilicate particles and Al_2O_3-coated SiO_2 nanoparticles. Plasma membrane proteome enrichment provided by the Fe_3O_4/Al_2O_3 pellicles was statistically significant and all three cationic nanoparticle pellicles (Fe_3O_4/Al_2O_3, aluminosilicate, and SiO_2/Al_2O_3) exhibited a strong enhancement of plasma membrane and transmembrane protein content relative to proteins in the whole cell lysate as classified by UniProt annotation and the web-based TMHMM tool [50]. Plasma membrane proteins represented 27.6% of the sample detected by the Fe_3O_4/Al_2O_3 pellicles method and 12% were transmembrane proteins, highest among the methods compared in this study [50].

Colloidal silica coupling relies on interactions between the negatively charged membranes to cationic colloidal particles. The method involves washing the cells with ice-cold MES-buffered saline followed by adding an ice-cold silica bead solution and incubating (for adherent cells) or gentle rocking (for suspension cells) prior to cell lysis, density gradient centrifugation, and protein purification [51].

Cationic colloidal silica particles based proteomics techniques have been utilized for the isolation of plasma membranes obtained from intact syncytiotrophoblast of human placenta [52], resulting in detection of 340 nonredundant proteins in the apical plasma membrane fraction. Proteins not previously known to be in the plasma membrane in the syncytiotrophoblast of human placenta include myosin proteins (11, 14, Ib, Id, Ie), nicalin, flotillin-1, and receptor expression enhancing protein 5 [52].

A cationic silica-polyanion (CSP) strategy was used to enrich plasma membrane from freshly isolated C57 mouse hepatocytes [53]. CSP was shown to provide a better yield compared with the biotin-avidin method. The CSP method yielded 185 nonredundant proteins while biotin-avidin isolated 49 nonredundant proteins, with 45 proteins identified by both methods [53].

A density perturbation technique modified from the silica nanoparticles subfraction strategy for in vivo studies in combination with MS was used to identify the surface proteome of liver sinusoidal endothelial cells [54]. The proteins were separated by SDS-PAGE and identified by with LC-MS/MS (GeLC-MS/MS). A total of 837 different proteins were found including 450 with membrane origin, in addition to contaminants from mitochondria, ribosomes, and the nucleus [54].

Although the progress in target discovery using proteomics has been substantial, serious challenges still remain. A large gap exists between the emerging new targets from abundant proteomics studies on various diseases and actual drug development, along with a lack of validation of specific roles played by these targets in the diseases. We believe that validation should incorporate more functional studies such as in vitro gene expression manipulation and gene knockouts along with clinical validation of identified novel targets. The recent advancements of targeted proteomics approaches such as PSAQ (protein standard for absolute quantification) and MRM techniques will hopefully accelerate both the validation and usage of proteomics targets and biomarkers in the pipeline of drug discovery, development, and clinical evaluation.

3 Biomarker discovery

Identification of both cell surface and serum markers is important for diagnostic and prognostic purposes when treating a disease. With the development of improved mass spectrometric techniques [55–60], cell surface and secreted proteins are more accessible for characterization. Analysis of cell surface protein is often made difficult by the low abundance of these proteins; however the recent application of plasma membrane enrichment [61] and glycoproteome enrichment [44,62,63] techniques have been helpful for the identification of greater numbers of potential biomarkers.

Serum biomarker discovery can be enhanced by the removal of high abundance proteins, such as albumin and immunoglobulins, so that low abundance proteins can be detected. One approach for serum depletion is to use commercially available kits such as the multiple affinity removal system (MARS—Agilent, Santa Clara, CA) spin columns or the Amersham (GE Healthcare, Piscataway, NJ) albumin and IgG removal kit [64].

Holewinski et al. tested a commercially available affinity capture reagent from Protea Biosciences and compared its efficiency and reproducibility to four other commercially available albumin depletion methods [65]. Two methods showed an albumin depletion efficiency of more than 97% for both serum and cerebrospinal fluid. Using LC-MS/MS analyses, they subsequently found 45 novel proteins in serum [65] and also showed that albumin isolation from serum can eliminate the need for fractionation to lower sample processing time [65].

Another study reported a method that coupled denaturing ultrafiltration with reverse phase fractionation and MS for characterization of low-molecular-weight proteins and peptides in serum and plasma samples [66]. The enriched peptides were analyzed by RP-LC combined

with MALDI-MS/MS characterization of the analytes, resulting in identification of 250 native peptides from 50 different proteins [66].

A study focused on exploring protein biomarkers in serum from rheumatoid arthritis patients treated with infliximab, an anti-tumor necrosis factor monoclonal antibody, employed a quantitative proteomics approach using 8-plex iTRAQ labeling [67]. A combination of depletion of the most abundant serum proteins, 2D-LC fractionation, protein identification, and relative quantification with a hybrid Orbitrap mass spectrometer was used to identify 235 proteins with high confidence [67]. Fourteen proteins that were significantly abundant in nonresponder patients as compared to the responder patients were identified as potential biomarkers. Some of the proteins showing significant changes and thus representing potential biomarkers were C4B-alpha chain, complement factor H-related protein 4, mannan-binding lectin, serine protease 2, and inter alpha trypsin inhibitor heavy chain H1 and H2 [67].

A plasma-proteomics based approach employing an 8-plex iTRAQ technique was used to study the association between nutrients and proteins in the plasma of 500 subjects after immune-depletion of six high abundance proteins including albumin and transferrin using an affinity removal LC column [68]. More than 4700 nonredundant proteins were identified including more than 450 proteins identified as extracellular, secreted, membrane, or lipoprotein associated. A strong correlation between vitamin A and retinol binding protein 4 (RBP4) was observed. Overall, the method demonstrated an approach for elucidating and quantifying protein biomarkers related to the nutrition environment [68].

In another study, Miltenyi Biotech’s MACS LS column was used for proteomic analysis of human prostate cancer cell line DU145 in order to identify potential biomarkers [69]. The DU154 prostate cancer cell line was isolated into CD44+ or CD44− cells using MACS and subsequent analysis of the differential expression of proteins between CD44+ and CD44− was carried out using 2DE and LC-MS/MS. The proteins cofilin and annexin A5, associated with proliferation or metastasis in cancer, were found to be positively correlated with CD44 expression [69].

Development of glycoproteome enrichment techniques has been highly effective for biomarker discovery through a comparison of the biologically significant glycoproteins between the diseased and normal subjects. However, development of user-friendly bioinformatics tools is required in order to enhance structural and compositional analysis of glycans. For efficient screening of biomarkers, precise quantification of glycosylation site occupancy at both relative and absolute levels is required. Indeed the recent advances in high-throughput glycoproteomics should provide new leads for potential biomarker identification.

4 Understanding the mechanism of action of drugs and disease

A substantial number of biological drugs and monoclonal antibodies affect signaling and phosphorylation events where they engage cell surface receptors. For this reason, the identification and quantification of protein phosphorylation sites are important areas of

study in the treatment of cancer and other diseases, such as diabetes, with biologic drugs. For example, identifying phosphotyrosine site occupancy can help in the diagnosis of these diseases [70–74] and provide a better understanding of the behavior of target cells upon treatment with drugs [75]. For this reason, a variety of phosphoproteomics techniques such as application of antiphosphotyrosine affinity chromatography, immobilized metal affinity chromatography, and TiO2 enrichment columns [72, 76] have been used to study the changes of phospho-peptide and phospho-protein states in cells.

Protein phosphorylation facilitates information transfer in cells and changes can contribute to the pathophysiology of diseases including cancer, inflammation, and metabolic disorders [13, 77–79]. One study investigated phosphorylation of serine, threonine, tyrosine, and histidine residues by protein kinases and reported a strategy using peptide arrays and motif-specific antibodies to identify and characterize substrate sequences for protein kinase A (PKA) [80]. It was found that protein kinase D and microtubule-associated protein-regulating kinase 3 can both be regulated by PKA. They also showed that the adaptor protein RIL is a PKA substrate that is phosphorylated on serine, which was predicted to regulate cell growth [80].

In order to understand the relationship between pancreatic cancer and alterations in proteomics and signaling pathways, a SILAC-based quantitative proteomics approach was employed to compare cells from three sites of metastasis (liver, lung, and peritoneum), which provided the proteome and tyrosine phosphoproteome of these tumors [81]. Approximately 42% of the proteome was found to be highly variable when compared across any two sites of metastasis in terms of receptor and signal transduction activity, suggesting that regulation of these tumors was different depending upon anatomical location [81].

Using a computational approach, in silico motif sequences were generated and compared with an experimental database—the Human Protein Reference Database (HPRD). Two hundred seven phosphotyrosine and 960 phosphoserine/threonine motifs were determined by this method [82]. The HPRD [83, 84] along with Human proteinpedia [85, 86] are centralized resources for phosphorylation site analyses and can be applied in a systems biology approach to determine the role of protein phosphorylation in protein function, cell signaling, biological processes, and their implications in the human diseases [87]. It contains experimental human phosphoproteome data from various methods including ^{32}P-labeled ATP followed by SDS-PAGE or HPLC followed by Edman sequencing to determine the site of phosphorylation [88]; phosphoproteome enrichment and LC-MS [70, 89–94]; high throughput analysis such as SILAC combined with titanium dioxide chromatography or IMAC microspheres for phosphoproteome enrichment [95–98]; or using pTyr-100 and 4G10 phosphotyrosine antibodies to enrich the phosphotyrosine proteome [99].

More work is required for the quantification of the phosphoproteome, as currently the work is mostly focused merely on identifying the phophosites.

5 Exosomal proteome profiling

With the improvement in exosome purification strategies together with MS-based proteomics tools with higher sensitivity and mass accuracy, exosomal profiling has proven to be a useful technique in studying disease states including cancer [100–117], AIDS [118, 119], Leishmaniasis [120], age-related macular degeneration [121], and neurodegenerative diseases [122]. Interestingly, the exosomal proteome is comprised of proteins found both in membrane and cytosol as well proteins with distinct functions in different cells. Proteomics has been very useful in cataloging the exosomes (extracellular membrane vesicles) that are versatile mediators of intercellular communication, pathogenesis, and might be useful for drug and gene delivery to a variety of tissues including cerebrospinal fluid [123], hepatocytes [124], and glioblastomas [125].

Exosomes derived from several types of tumor cells such as breast adenocarcinoma [126], colorectal cancer [127, 128], mammary adenocarcinoma [129], melanoma [130,131], mesothelioma [132], and brain tumor [101, 133] have been characterized by isolation strategies including differential centrifugation, filtration, sucrose density gradient, and immunobeads, combined with MS-based proteomic strategies such as 1D and 2D LC-MS/MS and MALDI-TOF/TOF MS [134]. A comprehensive review of studies that have used proteomic methods to characterize exosomes derived from in vitro sources and biological fluids has been previously reported [135].

Various methods for exosomes' isolation [136] include:

1. Ultracentrifugation: Requires centrifugation at 100 000 × *g* followed by resuspension in PBS and recentrifugation.
2. Size exclusion chromatography: Involves application of samples to a 2% agarose-based gel column and eluting isocratically with PBS.
3. Magnetic beads: Requires adsorption to anti-EpCAM antibodies coupled to magnetic microbeads followed by magnetic separation that results in the exosomes attached to the magnetic beads. The magnetic beads can be washed with TBS and exosomes can be extracted using a Trizol extraction protocol.

ExoQuick™ precipitation is another widely used exosomes isolation method that involves addition of ExoQuick™ precipitation solution and centrifugation. When the supernatant is aspirated, the exosomal pellet can be extracted using Trizol extraction procedures. While the mechanism of action of ExoQuick™ is not disclosed by the manufacturer, it is commonly used by some researchers [137] and can provide high yields and purity when compared to other methods [138].

In one study, ultracentrifugation and filtration techniques were used for isolating exosomes from human neuroblastoma cell lines for proteomic analysis [139]. A discrete set of molecules including tetraspanins, prominin-1 (CD133), and basigin (CD147) were found to be expressed in the exosomes that suggests the important role of exosomes in the modulation of the tumor microenvironment and indicates their potential utilization as a tumor biomarker [139].

Ultracentrifugation was used for exploring the urinary exosome in Zucker diabetic fatty rats to study diabetic nephropathy and related renal disease [140]. Two-hundred eighty-six proteins comprised mainly of membrane proteins were identified with many associated with functions such as transport, cellular communication, and cellular adhesion. A renal protein, Xaa-pro dipeptidase, which is linked with collagen breakdown was found to be upregulated and major urinary protein 1 was downregulated. These differentially regulated proteins may represent biomarkers for understanding metabolic changes during the progression of diabetes in obese diabetic mice models [140]. In another study, the proteome of exosomes shed by myeloid-derived suppressor cells (MDSC) was explored using ultracentrifugation [141]. Under an increased inflammatory response, proteins such as GTP and ATP-binding proteins (ATP-citrate synthase, ADP-ribosylation factor 1) were found to be in high abundance by MS analysis. Additionally, proinflammatory proteins S100A8 and S100A9 were found to be abundant in MDSC-derived exosomes [141].

One of the challenges in isolating low-density exosomes by ultracentrifugation is the variability between runs. Although relatively large starting volumes can be used [142], issues are still present for actual clinical samples that are low in volume. The exosomes can also be trapped on antibody-coated magnetic beads that have advantages such as immunoblotting, flow cytometry, and electron microscopy analysis of bead-exosome complexes, but this method is problematic for large sample volumes [143]. Size-exclusion chromatography, ultracentrifugation, and ExoQuick™ precipitation are not useful for preferentially isolating exosomes such as tumor-specific derived exosomes, which require immunoaffinity-based approaches [138]. In coming years, an improvement in MS-based proteomics and exosomal isolation and enrichment methods will shed light on fundamental biology questions such as the signaling pathways activated by exosomes in different diseases.

6 Secretome profiling

The secretome can be isolated from serum-starved cells or SILAC labeling can be used to quantitatively compare the secreted proteins of different cells [144]. A study on the secretome of differentiating primary adipocytes resulted in identification of 420 differentially expressed proteins including collagen triple helix repeat containing 1, cytokine receptor-like factor 1, glypican-1, hepatoma-derived growth factor, SPARC-related modular calcium-binding protein 1, SPOCK 1, and sushi repeat-containing protein [145].

In another report, SILAC-based quantitative proteomics was employed to elucidate the differences in the secretome of neoplastic and nonneoplastic gastric epithelial cells [144]. Out of 263 overexpressed proteins in cancer-driven cell lines, three novel proteins candidates —proprotein convertase subtilisin/kexin type 9 (PCSK9) with 13-fold overexpression, lectin mannose binding 2 (LMAN2) with sixfold overexpression, and PDGFA associated protein 1 (PDAP1) with 5.2-fold overexpression, represent possible biomarkers for the progression of gastric cancer [144].

7 mAb discovery

Proteomics methods are now being extensively incorporated into the mAb discovery process as a supplement to traditional hybridoma and phage display technologies. Indeed, a proteomics approach based on LC-MS/MS for identifying an antigen-specific antibody from sera and B-cell sources of immunized rabbits and mice was coupled with next-generation sequencing approaches [146]. This approach combined proteomics with enrichment of polyclonal antibodies via an affinity purification method. The enriched polyclonal antibodies were subsequently analyzed by LC-MS/MS. This integrated approach four rabbit antibodies and one mouse antibody [146]. Wine et al. also used antigen-affinity chromatography for the enrichment of antibodies followed by LC-high resolution MS/MS for discovering the antigen-specific antibody composition of a polyclonal serum response after immunization [147].

8 Bioinformatics and proteomics

With large amounts of data generated by proteomics studies, software demands and opportunities are emerging to facilitate and enhance data analysis such as: (1) compatibility with MS technology to support the data format and fragmentation patterns, (2) methods for identification of peptides and posttranslationally modified peptides, (3) application of known peptide libraries, (4) algorithms that increase speed and quality of analysis, and (5) user-friendly formats all of which will facilitate the widespread use of proteomics. Some of the most commonly used search engines for peptide and protein identification for MS-based data are Mascot [148], X! Tandem [149], SEQUEST [150], MyriMatch [http://forge.fenchurch.mc.vanderbilt.edu/scm/viewvc.php/*checkout*/trunk/doc/index.html?root=myrimatch.], and TagRecon [http://forge.fenchurch.mc.vanderbilt.edu/scm/viewvc.php/*checkout*/trunk/doc/index.html?root=tagrecon]. TagRecon allows users to identify proteins even with unexpected mutations and PTMs commonly observed in disease states [151]. After data analysis, visualization of the results is an important step toward interpretation. Most large software packages offer a variety of ways of visually displaying the data input and output, such as spectromania [152] that provides 2D, stacked chromatographic display of input spectra. It also allows a set of spectra from different packages to be merged into new and averaged spectra (with less noise). Other such visualization tools are MSight [153] and MassView [154].

A variety of bioinformatics tools are also available for biological interpretation of these high-throughput data. Tools such as KEGG (kyoto encyclopedia of genes and genomes) [155, 156], IPA (ingenuity pathway analysis—www.ingenuity.com), GO (gene ontology) [157], cytoscape [158], and DAVID (database for annotation, visualization, and integrated discovery) [159, 160] are widely used for interpretation of quantitative proteomics data. In addition to these, publically available software are present to address the localization of the proteins in a cell. For instance, TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) developed by the Center for Biological Sequence Analysis in Denmark uses a hidden Markov model in order to predict transmembrane helices of the membrane proteins [161]. Phobius (http://phobius.sbc.su.se/), similar software developed at the Center for Genomics

and Bioinformatics in the Karolinska Institute in Stockholm, Sweden [162], predicts signal peptides and regions of transmembrane protein sequence. In addition to Phobius, SignalP, and TargetP are two other bioinformatics tools used to predict the presence of signal peptides in a protein sequence. SignalP (http://www.cbs.dtu.dk/services/SignalP/) was developed at the Center for Biological Sequence Analysis in Denmark and is based on neural network algorithms. TargetP (http://www.cbs.dtu.dk/services/TargetP/) predicts signal peptides in protein sequences as well as their subcellular location with a success rate of 90% [163]. For identifying secreted proteins, SecretomeP 2.0 server (http://www.cbs.dtu.dk/services/SecretomeP/) uses neural networks to identify secreted proteins by nonclassical pathway i.e., the proteins not containing an N-terminal signal peptide [164]. WoLF PSORT (http://wolfpsort.org/) provides protein subcellular localization using PSORT features for prediction [165, 166]. Secreted Protein Database (SPD-http://spd.cbi.pku.edu.cn/) contains secreted proteins from human, mouse, and rat proteomes, including sequences from SwissProt, Trembl, Ensembl, and RefSeq [167].

Despite the tremendous progress in software applications, bioinformatics for MS-based proteomics still faces numerous needs such as user-friendly databases and programs, methods to reduce protein interference for quantification, tools for peptide level statistical analysis [168], and overall fully integrated software packages including capabilities provided by R (R Development Core Team 2008) [169]. With development and use of cloud-based computational resources, more proteomic data is likely to be made publically available, thereby giving open-source platforms a boost. In the future, MS instrumentation is likely to become even faster, more robust, and more accurate; equally powerful and enhanced bioinformatics approaches will be required to keep pace.

9 Conclusions

The potential of proteomics is now being realized in order to decipher the pathophysiology of various diseases and to help in the design of novel treatment strategies. Due to significant improvements in technology, powerful proteomics capabilities are emerging to provide opportunities for the discovery of new biomarkers and targets for drug discovery and development. One of the critical barriers to discovery is the challenge associated with identifying biomarkers or targets that are present in low abundance. In this review, we describe a variety of the emerging technologies that can be applied to detect and characterize lower abundance proteins that may be important in biomedicine. Different analytical techniques such as ultracentrifugation, biotinylation, colloidal silica, size exclusion chromatography, ExoQuick™ precipitation, hydrazide chemistry, and titanium dioxide incubation have been coupled with LC-MS/MS in order to achieve greater coverage of the subcellular proteome. These approaches often facilitate enrichment of the membrane proteome, glycoproteome, phosphoproteome, secretome, and exosome.

However, there is still much work needed in interpreting the enormous amounts of data generated from these improved techniques and in the development of easy to use software and robust databases. With the increasing incorporation of new high-throughput technologies, tremendous opportunities exist to expand our capabilities for discovery of new

and important biomarkers and targets, all with the ultimate goal of better understanding disease and in developing better medicines to treat patients.

Abbreviations:

CSP	cationic silica-polyanion
FASP	filter-aided sample preparation
GAL	galactose oxidase and aniline-catalyzed oxime ligation
HPRD	Human Protein Reference Database
MDSC	myeloid-derived suppressor cells
PAL	periodate oxidation and aniline-catalyzed oxime ligation
PKA	protein kinase A
PDGFA	platelet-derived growth factor alpha polypeptide
SCID	severe combined immunodeficiency
SPARC	secreted protein, acidic, cysteine-rich
TMHMM	transmembrane hidden Markov model

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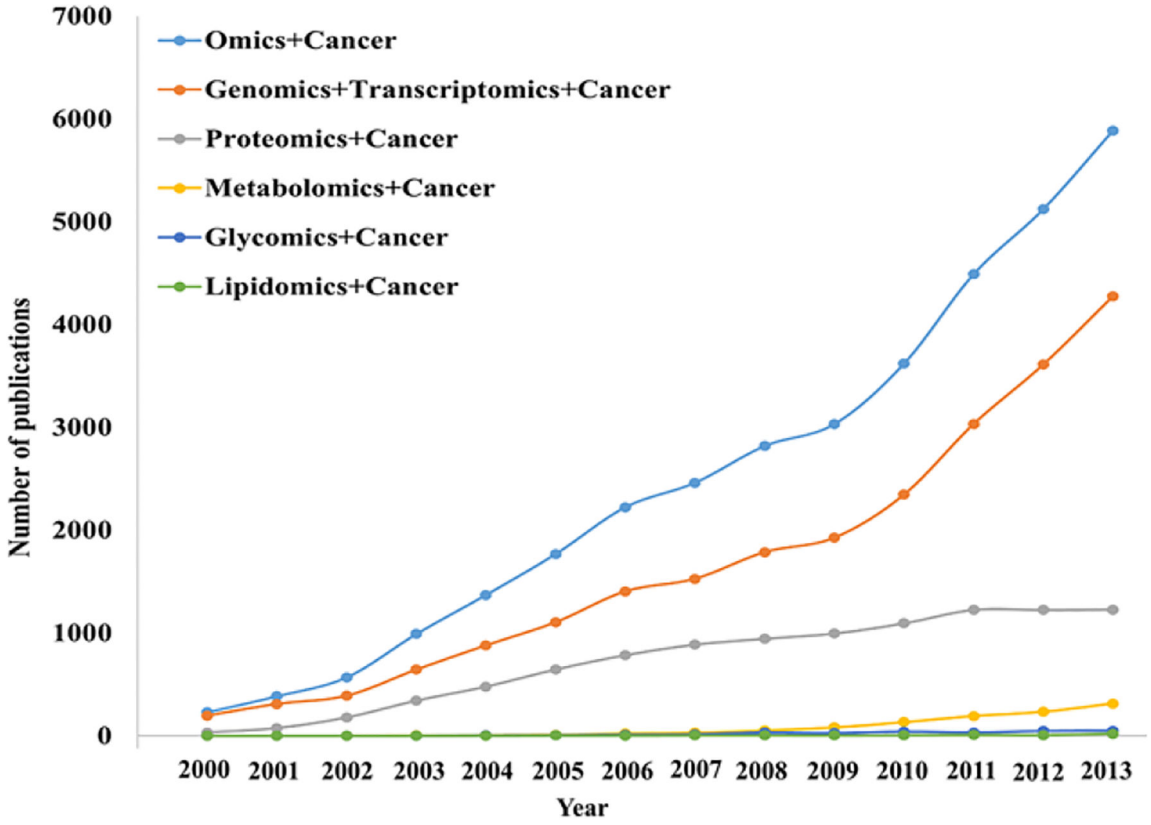

Figure 1.

Number of pubmed publications per year between 2000 and 2013 containing keywords "Omics and Cancer," "Genomics and Transcriptomics and Cancer," "Proteomics and Cancer," "Metabolomics and Cancer," "Glycomics and Cancer," and "Lipidomics and Cancer."

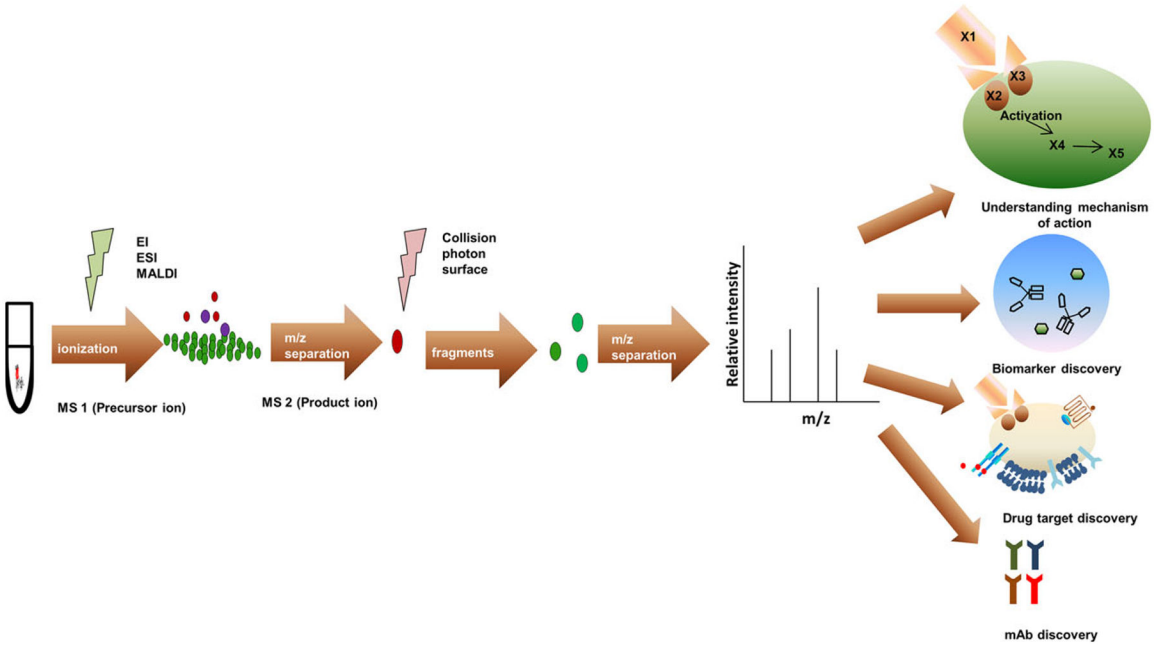

Figure 2.

Applications of MS proteomics methodologies include understanding mechanisms of drug action, identifying novel biomarkers, elucidating drug targets, and monoclonal antibody discovery.

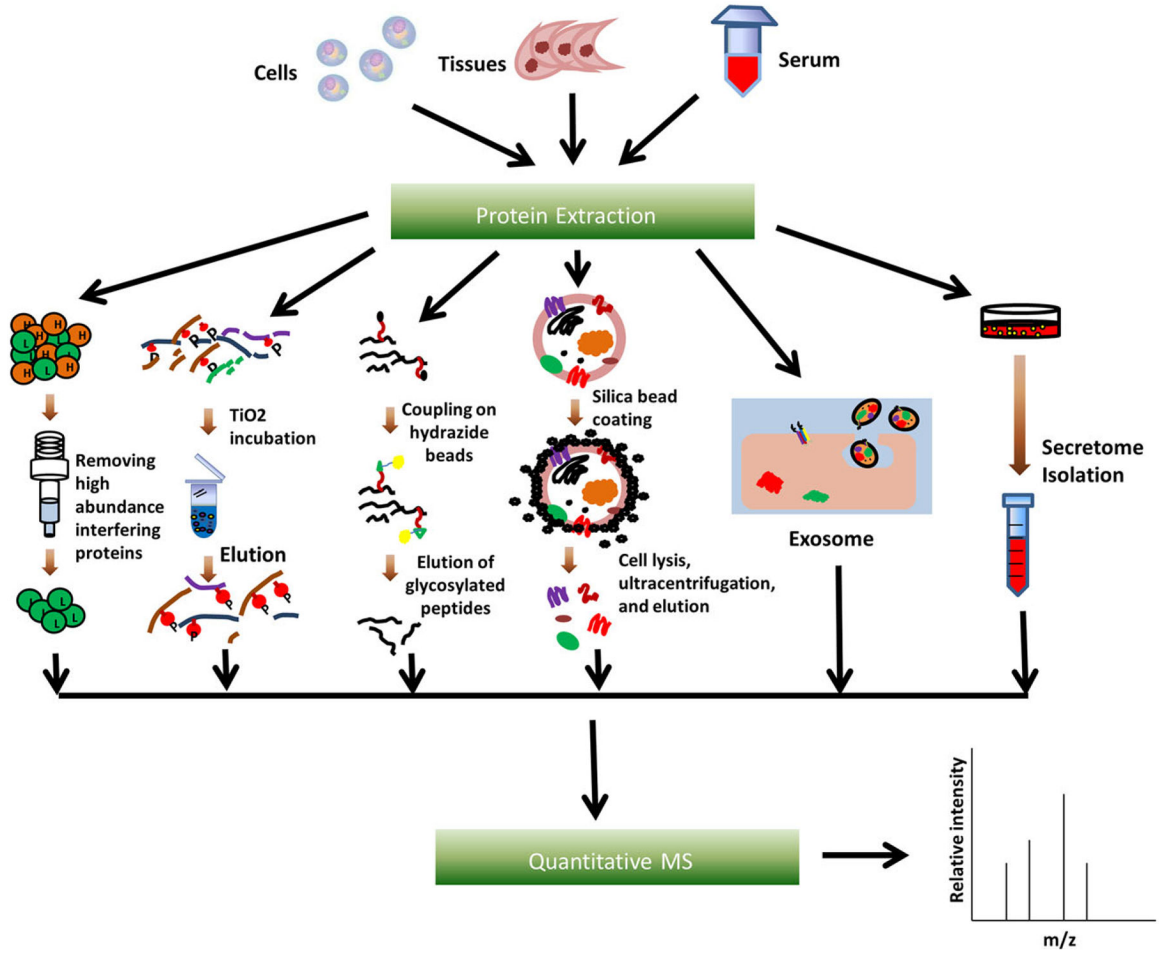

Figure 3.

Different techniques used in proteome profiling including (A) serum depletion method, (B) titanium dioxide incubation for phosphopeptides isolation, (C) glycopeptides capturing, (D) colloidal silica coupling for membrane proteins, (E) exosome isolation, and (F) secretome isolation.

Table 1.

A summary of various methods used in membrane proteome enrichment

Membrane enrichment method	Principle	Membrane protein percentage based on GO annotation	Transmembrane proteins percentage	Reference
Colloidal Silica	Electrostatic coupling between plasma membrane and cationic silica	65%	44% (UniProt and Human Protein Reference database)	[52]
Biotinylation	Affinity between biotin and avidin homologous proteins	51%	NA	[27]
Ultracentrifugation	Separation based on density	70%	NA	[24]
Glycoproteomics	Chemical coupling	41%	34% (KEGG pathways database)	[47]