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MINI-REVIEW

Proteomic-based biomarker discovery for development of next generation diagnostics

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Abstract In the post-genome age, proteomics is receiving significant attention because they provide an invaluable source of biological structures and functions at the protein level. The search for disease-specific biomarkers for diagnostic and/or therapeutic applications is one of the areas that proteomics is having a significant impact. Thus, the identification of a "good" biomarker enables a more accurate early diagnosis and prognosis of disease. Rapid advancements in mass spectrometry (MS) instrumentation, liquid chromatography MS (LCMS), protein microarray technology, and other protein profiling methodologies have a substantial expansion of our toolbox to identify disease-specific protein and peptide biomarkers. This review covers a selection of widely used proteomic technologies for biomarker discovery. In addition, we describe the most commonly used approaches for diagnosis based on proteomic biomarkers and further discuss trends and critical challenges during development of cost-effective rapid diagnostic tests and microfluidic diagnostic systems based on proteomic biomarkers.

Keywords Biomarker discovery · Proteomic · Diagnostic kits · Microfluidic systems

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Introduction

Proteomics is one of the most potent methods in biomedical research, which enables identification and comprehensive characterization of cellular targets and understanding the mechanisms of actions for therapeutic agents and the main functional constituents of biochemical schemes, specifically proteins (Bhalla et al. 2010). Mining genomes and mapping proteomes, the protein complements to genomes in cell and tissue, are being applied to develop and evaluate novel protein targets. They have been fully explored to discover the mechanisms of action of compounds and to identify novel markers for diagnostic and clinical uses (Veenstra and Smith 2003). Proteins expressed within a recognized proteome can be used to assess significant alterations in levels of biomarkers and their expressions in various pathological conditions. Proteomic profiling is expected to provide much needed insight into disease mechanisms and to bring forward therapeutic targeting candidates (Reisdorph et al. 2009; Seillier-Moiseiwitsch et al. 2002). The principal aim of current proteomics is to identify and characterize potential biomarkers by addressing two aspects: functional and expression proteomics. Functional proteomics deals with characterization of proteins in organelles and complexes, while the latter deals with measuring protein-level fluctuations under given conditions or parameters. Indeed, expression proteomics can serve as a powerful tool to identify changes in protein

expressions in disease state or during treatment in response to drug therapy. An approach often used for enrichment of low-abundance proteins involves isolation of subcellular compartments. Through methods of subcellular fractionation, it is possible to isolate individual organelles and then employ their proteins to identify essential protein complements using proteomics. The method is beneficial to study specific subcellular organelles that are associated with particular disease types (Morand et al. 2005).

Rapid detection and treatment serves as strong tool to prevent and control the progression of infectious diseases. A panel of biomarkers can be made into commercial diagnostic kits and offer better proficiency with regard to precision and economy (Khalilpour et al. 2013; Karami et al. 2006a, b; Karami et al. 2008; Yakovleva et al. 2002). This review provides an overview of the most commonly used techniques in biomarker discovery. It also explores the role of protein biomarkers in development of diagnostic kits and microfluidic technologies and briefly outlines future directions in this dynamic and promising field.

Biomarker discovery

Diagnostic markers are the main cellular or molecular events that link a specific environmental exposure to a health outcome. Diagnostic markers show associations between exposure to environmental compounds, diagnosis of subgroups that are at increased risk for disease, and development of chronic diseases. Great progress has been achieved in recognizing and evaluating new biomarkers that can be applied in population-based studies of environmental epidemiology (Madu and Lu 2010). The first biomarker was reported in 1948, where light chains of immunoglobulin in urine samples of more than 70% of patients with myeloma were detected. This protein is still used today and new methods also developed to detect, identify, and quantify this protein for more accurate and reliable diagnosis (Allred et al. 1998; Kulasingam and Diamandis 2008). From 1930 to 1946, a number of proteins were recognized in biological systems from cancer patients; however, monitoring of malignant diseases fundamentally began with the diagnosis carcinoembryonic antigen and alpha-fetoprotein in the 1960s. The original method employed the quantification of radioisotopes, but methods developed later replaced it with enzyme immunoassays in the 1980s (Francis and Stein 2015; Marzese et al. 2013). Biomarker detection in cancer and infectious diseases has become more popular, and thousands of diagnostic markers have been introduced to detect at its earliest manifestation. Nonetheless, only a few of these biomarkers have passed high bars to attain the status of surrogate endpoints for routine clinical application. This may partially be associated with technical challenges in the testing itself. But in most conditions, it is attributable to overlay of variations between normal and cancer patients, which cannot be easily distinguished from each other. Most tumor-associated markers display significant upregulation in abnormal cells with increased levels of protein expression. However, many of these biomarkers such as prostate-specific antigen (PSA), cancer antigen 15.2, carbohydrate antigen 19.9, and cancer antigen 125 suffer from lack of sensitivity and specificity and do not correlate with classic tumor prognostic factors (Elshimali et al. 2013; Francis and Stein 2015). Numerous protein detection and quantitation methods for biomarker discovery have been devised. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting are the most basic and traditional techniques used for separation of macromolecules. Currently, these techniques have been combined with high-throughput techniques. Different experimental methods, such as two-dimensional electrophoresis (2-DE), bioinformatics software, mass spectrometry (MS)-based high-throughput proteomics, and high-performance liquid chromatography (HPLC), have been widely employed in all areas of disease biomarker discovery.

High-throughput techniques for biomarker discovery

Diagnostic markers play major roles in molecular medicine in the identification, validation, early diagnosis, disease prevention, and drug target identification. There are numerous protein detection methods that have been utilized in biomarker discovery. In this review, an overview of some of the essential tools for effective protein marker analysis in medical diagnostic devices has been provided. However, describing these technologies in depth goes beyond the scope of this review. Figure 1 provides the general workflows in proteomics, and we addressed their advantages and disadvantages in Table 1.

2-DE

Most proteomic protocols integrate 2-DE for antigen separation, where antigens are identified with various stains, and protein profile is then analyzed using 2-D gel analysis software (Ge et al. 2003; Molloy et al. 2000, 1998). 2-DE combines isoelectric focusing (IEF) and SDS-PAGE in the first and second separation dimensions. This combination is to isolate proteins based on isoelectric point (pI) molecular mass (M_r), the two factors which are involved in the first and the second dimension separations, respectively (Fig. 2a) (Rabilloud and Lelong 2011). Alternative 2-DE methods, such as incorporating SDS-PAGE with PAGE systems based on the application of cationic detergents like cetyltrimethylammonium bromide (CTAB) (Macfarlane 1989) or benzyldimethylamine-hexadecylammonium chloride (BAC) (Macfarlane 1989), enable two-dimensional separation of hydrophobic membrane proteins, and they have received more attention recently

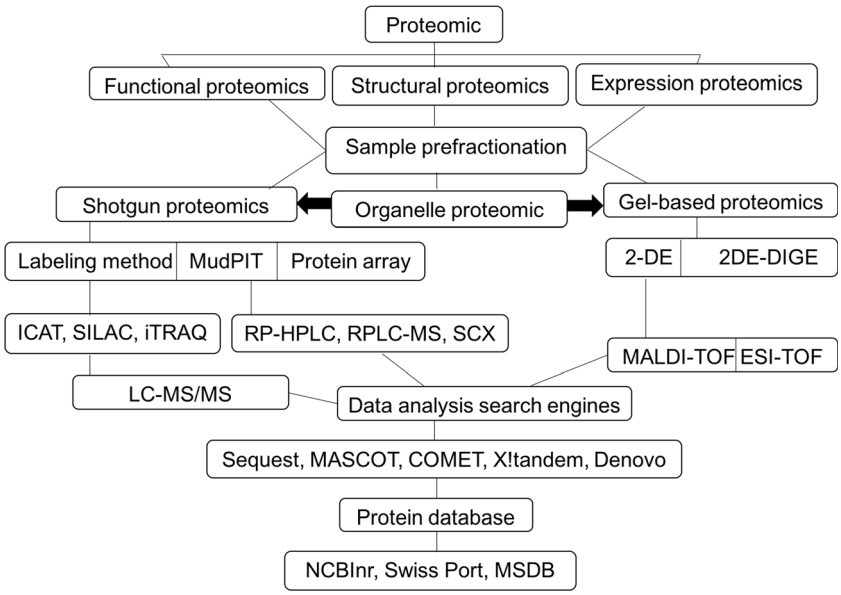


Fig. 1 Schematic of proteomic strategy

(Bertrand and Faupel 2007). On the other hand, 2-DE may not be feasible for general characterization of extremely intricate proteomes due to their exceptional physicochemical characteristics. In spite of technical challenges of 2-DE, it is still the most common technique for diagnostic research and will most likely remain so in near future (Gorg et al. 2004). High-resolution 2-DE can identify up to 4000 different proteins simultaneously and detect and measure <1 ng of protein per spot. Recent improvements permit analysis on a single 2-DE gel containing mixed samples differentially labeled by fluorescent dye molecules using difference gel electrophoresis (DIGE) technology (Viswanathan et al. 2006). The important aspect of the DIGE technology is its ability to label all proteins in a given sample with one set of complemented fluorescent dyes intended to enable 2-DE analysis with minimal protein mobility interference (Unlü et al. 1997).

Two types of fluorescent dyes—namely, CyDye® minimal dyes and CyDye saturation dyes—are available for use in DIGE analysis. CyDye minimal dyes react with the NHS ester bond of lysine ε-amino residues and thus accommodate the co-electrophoresis of three different samples per assay. For particular applications, including samples from microdissection, the CyDye saturation containing a maleimide group reacts with the cysteine residues being exposed on the surface of protein, and this results in highly concentrated labeling. Thus, the process facilitates the full 2-D analysis and quantification of protein abundance changes in sample quantities involving extremely small quantities (Alban et al. 2003; Gharbi et al. 2002; Knowles et al. 2003; Tonge et al. 2001).

OFFGEL electrophoresis fractionator

The OFFGEL electrophoresis fractionator is a novel technique that is commercially offered by Agilent Technologies. It offers an effective separation method to isolate peptides or proteins recovered through immobilized pH gradient (IPG) strips (12-well or 24-well IPG gel strip of pH 3–10 or pH 4–7) solution according to their isoelectric points (Fig. 2b) (Heller et al. 2005; Michel et al. 2003). Consequently, its microscale sample size affords fraction volumes large enough so that subsequent analysis like reverse phase (RP)–liquid chromatography (LC)–matrix-assisted laser desorption and ionization (MALDI) MS/MS can be performed (Chenau et al. 2008). The protein IEF by OFFGEL has a resolution of at least 0.3 pH units using a linear pH gradient of 3–10, and the proteins can be recovered in solution form with high reproducibility and accuracy in high yield resolve proteins at 0.15 pH units (Heller et al. 2005). The second dimension is performed in the same way as in the regular SDS-PAGE method, where an anionic detergent SDS that denatures proteins is used (Lodish et al. 2000a; Lodish et al. 2000b). In SDS-PAGE analysis, proteins are separated based on molecular size; smaller molecules move faster and migrate farther than larger ones in polyacrylamide gel by applying an electric field (Khalilpour et al. 2013; Moghadam et al. 2013; Khalilpour et al. 2012; Maghsoudi et al. 2007). Following electrophoresis, the gel is treated with Coomassie Brilliant Blue solution or subjected to silver staining (Fig. 2c). Finally, the gel image is taken by an image analyzer system (such as Fluorochem) and the gel is dried using a dryer for long-term storage. The molecular weight of the target protein is determined by comparing to the values obtained for molecular weight standards (Das et al. 2011).

The OFFGEL technology has several advantages such as possibility of liquid phase protein recovery (Abdallah et al. 2012; Ros et al. 2002), high resolution, buffering capacity, and high sample loading (Rabilloud et al. 2009). A few disadvantages of OFFGEL devices include long separation time, requirement of high amount of protein for off-gel

Table 1 Common technologies used in proteomic studies, applications, advantages, and their disadvantages

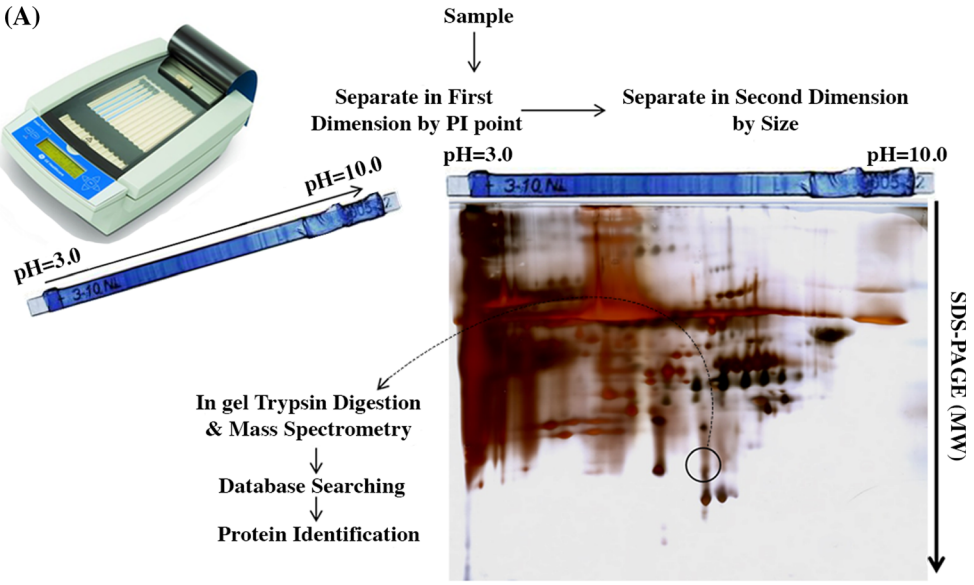
Technology	Application	Advantages	Disadvantages
SDS-PAGE	Protein separation	Compatible with SDS and other ionic detergents in sample buffer	Presence of several proteins in a gel band No information about PTMs (only M_r, no pI)
2-DE	Protein separation Quantitative expression profiling	Very sensitive High resolution Relative quantitative PTM information (M_r, pI)	Sample preparation Problems to analyze hydrophobic, high M_r, very basic proteins
DIGE	Protein separation Quantitative expression profiling	Relative quantitative PTM information High sensitivity Reduction of intergel variability	Proteins without lysine cannot be labeled Requires special equipment for visualization and fluorophores are very expensive
ICAT	Chemical isotope labeling for quantitative proteomics	Sensitive and reproducible Detect peptides with low expression levels	Proteins without cysteine residues and acidic proteins are not detected
SILAC	Direct isotope labeling of cells Differential expression pattern	Degree of labeling is very high Quantitation is straightforward	SILAC labeling of tissue samples is not possible
iTRAQ	Isobaric tagging of peptides	Multiplex several samples Relative quantification high throughput	Increases sample complexity Require fractionation of peptides before MS
MUDPIT	Identification of protein-protein interactions Deconvolve complex sets of proteins	High separation Large protein complex identification	Not quantitative Difficulty in analyzing the huge data set Difficult to identify isoforms
Protein array	Quantitate specific proteins used in diagnostics (biomarkers or antibody detection) and discovery research	High throughput Highly sensitive Low sample consumption, fast, easy control of experimental condition	Limited protein production Poor expression methods Availability of the antibodies Accessing very large numbers of affinity reagents Difficulty to control PTMs
Mass spectrometry	Primary tool for protein identification and characterization	High sensitivity and specificity high throughput Qualitative and quantitative PTM information	No individual method to identify all proteins. Not sensitive enough to identify minor or weak spots. MALDI and ESI do not favor identification of hydrophobic peptides and basic peptides
LC-MS/MS	Protein separation	Very high sensitivity Allows analysis of membrane proteins	Lack of pI and M_r estimate of proteins Requires significant computing resources for data analysis
Bioinformatics	Analysis of qualitative and quantitative proteomic data	Functional analysis, data mining, and knowledge discovery from mass spectrometric data	No integrated pipeline for processing and analysis of complex data. Search engines do not yield identical results

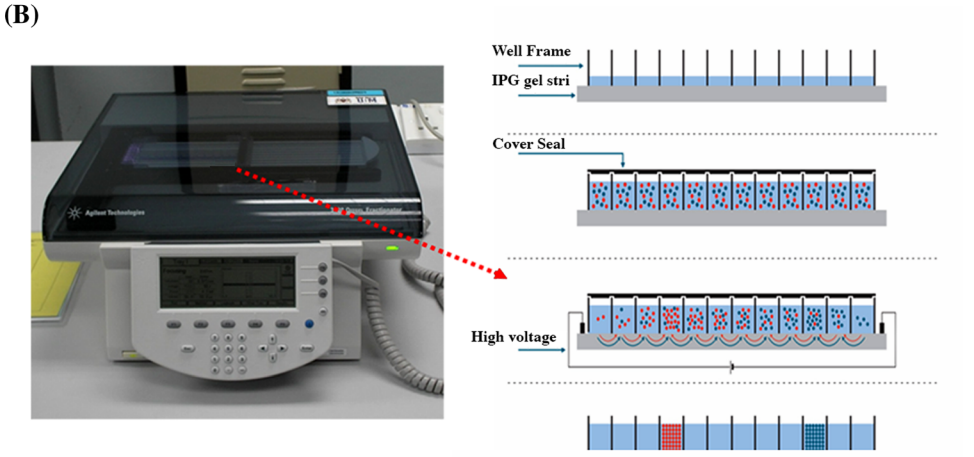
fractionation, and loss of protein when doing in-gel IEF (Khalilpour et al. 2013; Moreda-Pineiro et al. 2014). The running time takes from a few hours to 2–4 days relevant to the complexity of the proteins in the sample, which is a quite long period when compared to the contractual running time in HPLC and gel electrophoresis (1-D SDS) methods (Khalilpour et al. 2013; Moreda-Pineiro et al. 2014; Karami et al. 2006a, b; Karami et al. 2007).

Western blotting

Western blotting is probably the most important technique used following second dimension of 2-DE and OFFGEL electrophoresis to detect protein bands with accurate sensitivity and specificity (Lodish et al. 2000a). By using western blots, scientists have been able to identify specific target proteins from complex mixtures of proteins extracted from cells according to their molecular weights and types through gel electrophoresis (Moore 2009). Following electrophoresis, the SDS-PAGE gel is transmitted to a nitrocellulose membrane via Trans-Blot® SD Semi-Dry Transfer Cell. The membrane is then cut into strips (i.e., 3 mm wide) and incubated with different groups of sera as primary antibody solutions and

Fig. 2 Schematic of principle protein identification using 2-DE and OFFGEL. **a** Protein separation based on isoelectric point (pI) in the first dimension and molecular mass (M_r) in the second dimension using 2-DE. **b** First dimension of the 2-D gel electrophoresis using the OFFGEL apparatus. **c** Gel profile (12% SDS-PAGE) of OFFGEL fractions of antigens using the 3100 OFFGEL High Res Kit, pH 3–10 (*silver* staining). **d** Western blotting profile of antigens incubated with patient (S+) and control serum sample (S−)


(A)
Sample
Separate in First Dimension by PI point
Separate in Second Dimension by Size
pH=3.0
pH=10.0
3-10 NL
SDS-PAGE (MW)
In gel Trypsin Digestion & Mass Spectrometry
Database Searching
Protein Identification


(B)
Well Frame
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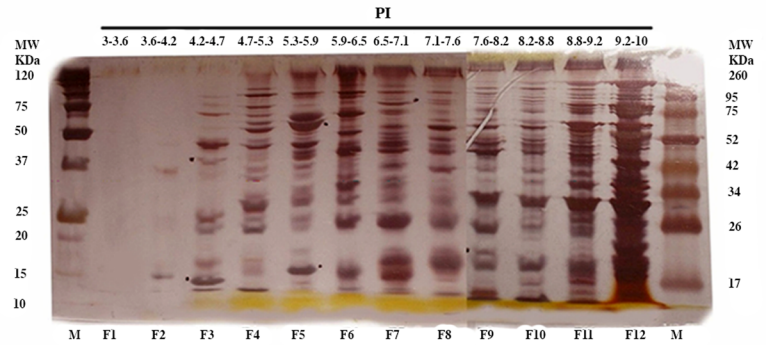
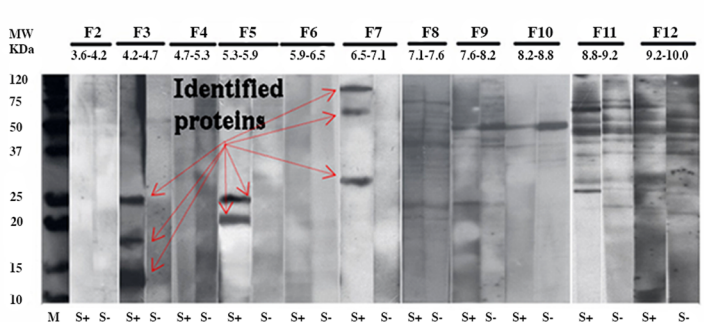

(C)
PI
3-3.6 3.6-4.2 4.2-4.7 4.7-5.3 5.3-5.9 5.9-6.5 6.5-7.1 7.1-7.6 7.6-8.2 8.2-8.8 8.8-9.2 9.2-10
MW KDa
120 75 50 37 25 20 15 10
260 95 75 52 42 34 26 17
M F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 F11 F12 M


(D)
MW KDa
F2 F3 F4 F5 F6 F7 F8 F9 F10 F11 F12
3.6-4.2 4.2-4.7 4.7-5.3 5.3-5.9 5.9-6.5 6.5-7.1 7.1-7.6 7.6-8.2 8.2-8.8 8.8-9.2 9.2-10.0
120 75 50 37 25 20 15 10
Identified proteins
M S+ S- S+ S- S+ S- S+ S- S+ S- S+ S- S+ S- S+ S- S+ S- S+ S- S+ S- S+ S-

secondary antibody, respectively. Finally, the strips are developed for visualization using a chemiluminescence substrate (Fig. 2d) (Khalilpour et al. 2013).

Mass spectrometry

Recently, two-dimensional polyacrylamide gel electrophoresis (2-D–PAGE) has been combined with mass spectrometry to achieve the direct and systematic identification of polypeptides (Han et al. 2008; Taylor and Johnson 2001). Generally, there are two main methods for protein identification: MS and Edman N-terminal sequencing (Egidi et al. 2014; Krijgsveld 2012). MS analysis of proteins has replaced classical methods for protein microsequencing (Weiss and Kim 2011), which has been widely used to analyze biological and clinical samples (Aebersold 2003; Han et al. 2008).

Every mass spectrometer comprises of three main components: (1) an ion source, (2) a mass analyzer, and (3) a detector. The function of ion source is to generate analyte ions. Among numerous ionization methods, MALDI and electrospray ionization (ESI) are most often employed in proteomics. The generated ions are then transmitted to the mass analyzer, where they are separated based on their mass-to-charge ratios (*m*/*z*). Different mass analyzers including MALDI time of flight (TOF), ESI-ion trap (IT), and ESI-Fourier transform ion cyclotron resonance (FTICR) can produce mass spectrums, in combination with ion sources (Inagaki and Katsuta 2004; Klose and Kobalz 1995). Additionally, more complicated experiments must be performed by MS in order to gain data on the primary construction of polypeptides. For example, specific data concerning amino acid sequences in polypeptides can be produced using mass spectrometers that allow for fragment ion analysis, gas-phase peptide fragmentation, and ion isolation (Michael Hamacher et al. 2006). Moreover, the sequence-specific fragmentation of insulated ions of peptides through means of collision-induced dissociation (CID) needs the paired application of two separate mass analyzers with either identical or distinct principles of ion separation. Such pairings are generally stated as tandem MS apparatuses. However, ion trap apparatuses and MALDI-TOF MS are exceptions to the rule. The ion trap apparatus facilitates successive sequences of peptide ion fragmentation in a single-stage device (Jonscher and Yates 1997), while peptide ions in the MALDI-TOF MSs are subjected to unimolecular decomposition taking place in a field-free drift tube, as a step usually stated as post-source decay (PSD) (Spengler 1997).

Bioinformatics in mass spectrometry data analysis

Selected gel bands from 2-DE or OFF-GEL section are cut out, digested, and extracted using an Agilent Protein In-gel Tryptic Digestion Kit. Subsequently, cleanup of the selected samples for MALDI-TOF/TOF mass analysis is done using Zip-Tip pipette tips containing immobilized resins such as C18 that attach at the head of the tip occupying about 0.5-μl volume (Khalilpour et al. 2013). The peptide that was digested from each gel band is mixed with 1.2 μl CHCA matrix solution (5 mg/ml cyano-4-hydroxy-cinnamic acid in 0.1% TFA, 50% ACN) and spotted onto a MALDI target plate. Peptide mass spectra are captured using the Proteomics Analyzer MALDI-TOF/TOF mass spectrometer. Databases from National Center for Biotechnology Information (NCBI) are used to analyze the results. The MS/MS and protein identification received data are then used to perform further analysis using the BLAST program (Tanca et al. 2013), which can be coupled with literature searches to further study the protein of interest.

For example, Khalilpour et al. (2014a, b) analyzed *Helicobacter pylori* samples using mass spectrometry. The samples were initially dissolved in 10 μl of 0.1% formic acid and transferred to a maximum recovery vial placed in the nano-ACQUITY sample manager. An injection of 1 μl was separated using a gradient of 0–40% acetonitrile over 40 min. The maximum MS peak intensity was around 3000 cps, showing optimal loading. Eluting peptides were analyzed in an automated MS/MSMS switching mode (DDA) using 1 MS/MS scan per MS scan (Khalilpour et al. 2016; Khalilpour et al. 2014a), and the criteria used to identify the protein were as follows: (1) the protein had significant scores in both protein report and peptide report, (2) there were more than one significant peptide in the peptide report, and (3) the same protein was identified from at least three gel slices from different experiments. The report provided a list of identified proteins and their scores (Fig. 3a). Several proteins could have significant scores. Thus, it is crucial to look at the peptide report further in order to correctly identify them (Fig. 3b) (Khalilpour 2016; Khalilpour et al. 2014a; Khalilpour et al. 2013; Nooradin et al. 2013). The protein with the top score (462) for a 25-kDa *H. pylori* band in fraction 5 (pI 5.33–5.90) of OFFGEL was urease acessory protein (UreG) of *H. pylori J99*. The score >83 indicates identity or extensive homology at a significant level ($p < 0.05$). Six significant peptides were matched to the amino sequence of UreG. The amino acids in red color were the peptides detected in the MALDI-TOF analysis that led to the identification of urease accessory protein (Fig. 3c). The molecular weight and isoelectric point were 22,098 Da and 5.02, respectively. These values were similar with the experimental results obtained, i.e., 25 kDa and pI between 5.33 and 5.90 (Khalilpour et al. 2014a; Khalilpour et al. 2013).

For MS analysis, over 1000 shots are usually gathered for each sample. MS information is routinely extracted from the five most strong ions designated for MS/MS (Saadatnia et al. 2011). The peptides are then exposed to MS/MS analysis using air with a clash energy of 2 kV and a collision gas pressure ~1×10^{-6} torr. Stop situations were performed so that 2000 to 3000 shots were gathered depending on the value

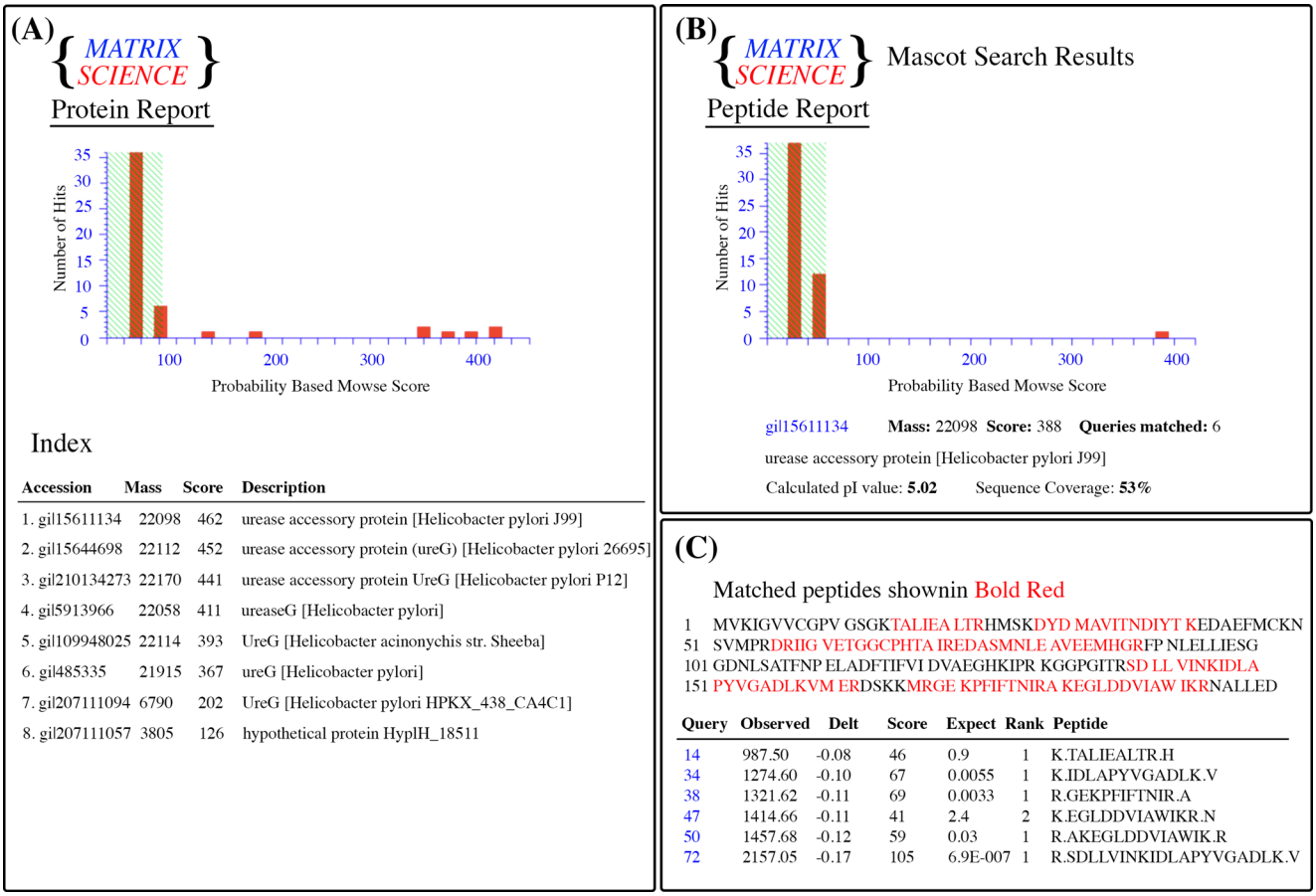


Fig. 3 Schematic of MASCOT research result. **a** MASCOT protein search result from MASCOT research engine, **b** the peptide summary report of identified protein, and **c** matched peptides of identified protein

of the spectra. The proteomic search engine (MASCOT) was applied to quest total tandem mass spectra (Khalilpour et al. 2014a; Khalilpour et al. 2013; Riazi et al. 2014). Zhao et al. (2015) used GPS Explorer software to quest files with the MASCOT search engine for protein and peptide identification. The search factors allowed for methionine oxidation, cysteine C-terminal carbamidomethylation, and N-terminal acetylation. Fragment mass tolerance and peptide mass tolerance were fixed to ±0.4 Da and 150 ppm, respectively (Zhao et al. 2015). Proteins were initially recognized using Protein Pilot proteomic software, and peptide masses were compared with a computer-generated database containing tryptic peptides of known proteins (Chen et al. 2014; Chen et al. 2015; Shirran and Botting 2010). Finally, the score which replicates the counterpart of the experimentally and theoretically indicated masses was assessed.

Liquid chromatography

Two major methods in proteomics are applied for the detection of proteins in intricate samples. The first method uses 2-D–PAGE to significantly decrease the intricacy of biological samples before peptide MS assay. The second method is one-dimensional gel electrophoresis (1-D PAGE), which provides a partial protein separation, and the result is added to online nano-HPLC/ESI-tandem MS resolution of peptides in a programmed procedure (Michael Hamacher et al. 2006). The latter method has been recognized for its efficacy in identification and/or quantification of proteins from complex mixtures (Link et al. 1997; Yates et al. 1996). In both chromatographic approaches, proteins are converted to a set of peptides using enzymatic assimilation before MS analysis (Michael Hamacher et al. 2006). In this case, the peptides—namely, those from membrane proteins—are solvable in various solvents, and therefore, they are easier to identify from integral proteins. However, this method also has some disadvantages by significantly decreasing the number of components in the mixture. Consequently, fractionation approach appears to be essential prior to mass spectroscopy.

The sample intricacy is further enhanced by protein assimilation, but the main causes for MS analysis of peptides instead of proteins are as the following: (i) MS has the greatest sensibility in detecting partial masses smaller than

2500 Da, (ii) instructive sequence data is gained from peptides of up to 20 amino acid residues by MS/MS, and (iii) peptides have higher solubility than proteins and consequently simpler to handle in order to chromatographically separate and electrospray them (Michael Hamacher et al. 2006). In regard to chromatography, these approaches use a bibasic column having a segment of reversed-phase substance supported by potent cation interchange resin. Recently, there has been some progress in utilizing these technologies in proteomics. For example, in another conversion, a third ingredient of reverse-phase substance can be combined to facilitate the online desalting of the sample. The benefits of this approach are greater automation, ability to evaluate membrane proteins, and mitigation of sample intricacy via consecutive steps and thereby allowing the mass spectrometer to recognize utmost or all of the components within its field of scope. This strategy has been enhanced during the past few years via the introduction of several tagging arrangements. Accordingly, isotope-coded affinity tag (ICAT) (Gygi and Aebersold 1999) and steady isotope tagging by amino acids in cell culture (SILAC) have become popular. The interpolation of steady isotopes into proteins authorizes the concurrent identification and quantification of proteins involved in two cellular conditions. However, while the SILAC technique is not appropriate for the investigation of clinical samples since isotope tagging is attained via a metabolic method, the ICAT and other chemical tagging processes employ post-extraction isotope tagging. This makes ICAT an appropriate choice of profiling and quantification for proteins analysis of clinical samples (Hamdan 2006).

Multidimensional protein identification technology

Multidimensional protein identification technology (MudPIT) is a non-gel method for identification of peptides/proteins in intricate combinations. As an alternative to the 2-DE technique, MudPIT (also known as shotgun proteomics) has proven to be very effectual for separating and identifying individual components of complex protein and peptide mixtures. It separates peptides in 2-D liquid chromatography compared to the traditional 2-DE. This allows greater separation of peptides, which can be directly interfaced with the ion source of a mass spectrometer and maximizes sensitivity (Yates 2016; Yates et al. 2000). In addition, MudPIT avoids the band broadening associated with many chromatographic steps, which can decrease resolution.

That being said, the MudPIT technology has some problems when the quantifying the significance of the peptides is considered (Aebersold and Mann 2003). For example, in the standard 2-D–PAGE method, numerous peptides of a certain protein typically approve the identification of that protein, while the MudPIT technique mostly contends identification of proteins based on two peptide sequence labels, which is a problem since the identical tryptic peptides can exist in partly different protein sequences. Thus, protein assignment can be more precisely determined using MudPIT method compared to other techniques (Beretov et al. 2014; Gevaert and Vandekerckhove 2000; Matthiesen 2007).

Isobaric tags for relative and absolute quantitation technology

Isobaric tags for relative and absolute quantitation (iTRAQ) is a quantitative technique suitable for comparative studies of normal, diseased, and drug-treated samples. This biomarker discovery method provides a quick and relative quantification of the changes in the proteome in complex mixtures using MS. Protein quantification through incorporation of stable isotopes has become the central technology in modern proteomic research. However, due to drastic increase in sample complexity, fractionation of peptides prior to MS is one of the iTRAQ limitations (Chandramouli and Qian 2009; Pan et al. 2009).

This technique contains several steps. First step is the preparation of samples under several treatment conditions. Then, the extraction of protein is prepared using cell lysis. To estimate the concentration of protein in each sample, production of proteolytic peptides is required. For this purpose, proteins are digested using an enzyme such as trypsin and labeled with a diverse iTRAQ reagent and combined into a combined peptide mixture. Then, the quantification and identification analyses of the sample are performed using LC-MS/MS (Fig. 4a) (Chandramouli and Qian 2009).

Protein microarray technology

In recent years microarray technology has become a central component in large-scale, high-throughput biology. This technology permits quick, simple, and parallel interrogation of thousands of addressable elements within a single experiment (Fig. 4b) (Hamdan 2006; Tao et al. 2007). Despite the relatively recent introduction of the concept, protein microarray technology has shown remarkable potential in regard to basic research, diagnostics, and biomarker discovery. However, some scientific barriers are yet to be overcome to realize the full impact of this emerging technology on proteomics, medical research, and drug/biomarker discovery. Nevertheless, the technology has demonstrated considerable potential. Therefore, it is reasonable to expect that technology advancements will bring further developments and improvements in this process, and it will be recognized as one of the most powerful tools in large-scale biology (Diez et al. 2012; Hall et al. 2007).

Thus, it is possible to investigate transcriptional patterns of several genes in various biological contexts simultaneously through microarray analysis (Diez et al. 2012). Nevertheless,

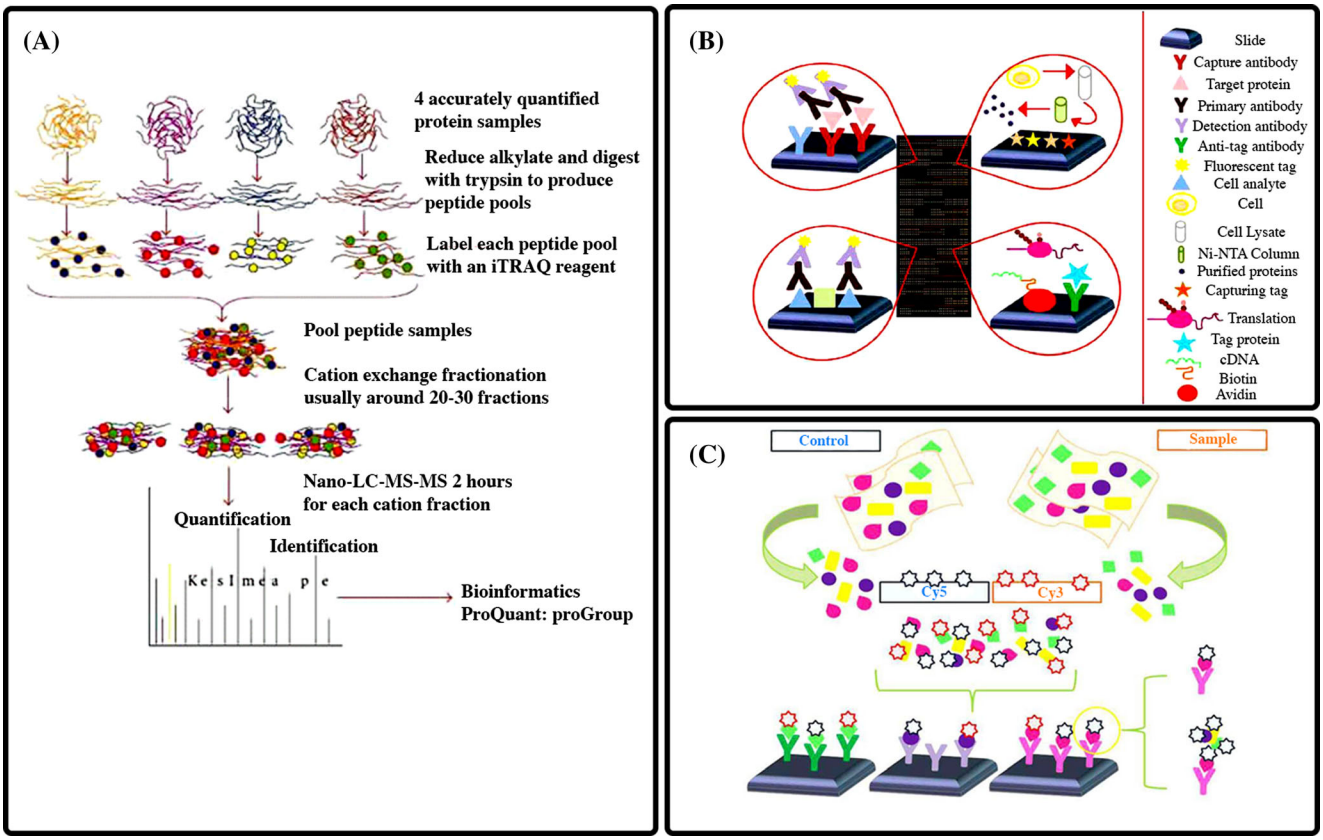


Fig. 4 Schematic of shotgun proteomic. **a** iTRAQ workflow. **b** Types of protein microarrays. (i) Capture arrays. (ii) Cell-based protein microarrays. (iii) Reverse phase arrays. (iv) Cell-free nucleic acid programmable protein array. **c** Microarrays for differential protein displays. Reprinted with permission from references Chandramouli and Qian (2009 and Diez et al. (2012)

transcriptional studies have some disadvantages. There is a less than predictable relationship between gene transcription and protein expression. By using biosynthetic labeling of RNA with uracil phosphoribosyltransferase, it allows microarray analysis of mRNA synthesis (Cleary et al. 2005). Furthermore, the activity of a protein is not only dependent on its abundance but also on its state of activation, often intermediated through a post-translational modification (PTM) event including phosphorylation.

Moreover, the activity of a protein is also dependent on its interaction with other proteins, so it is vital to identify and understand the properties of these interactions in analysis of protein function (Van Hoof et al. 2008). However, limited protein production, poor expression methods, availability of the antibodies, accessing very large numbers of affinity reagents, and difficulty to control PTMs are considered to be the main limitations of using protein microarray method.

Application of biomarker for the development of diagnostic systems

Diagnostic markers play major roles in molecular medicine in identification, validation, diagnosis, and prevention of diseases. Diagnostic markers can reflect biological activities that are relevant to disease and provide valuable information for diagnostic and therapeutic use. A variety of infection disease antigens using proteomic technologies have been reported to elicit strong humoral immune responses making them potential candidates for diagnostic markers. Table 2 shows some of the identified biomarkers from infection diseases (Glassman 1990; Khalilpour et al. 2016; Lee et al. 2010a).

Diagnostic rapid test

Two types of markers including nucleic acid (DNA and RNA) and protein markers are utilized in commercial kits for diagnosing infectious disorders. The precision of these markers varies from assay to assay and depends significantly on the variety of sample and test (el-Zaatari et al. 1997). Protein-based markers are very popular compared to the DNA-based markers, as it is manufactured into inexpensive user approachable forms, including immobilized strips. In addition, protein-based diagnostic kits are similarly accessible in ELISA and latex agglutination assay cards. Immunoblotting is also run as a lab-based serological technique. These tests discover antibodies in either fecal, whole blood, serum, or urine (Glassman et al. 1990; Miwa et al. 2001; Sasidharan and Uyub 2009; Simor et al. 1996; Zuniga-Noriega

Table 2 Lists of some biomarkers identified by proteomic technologies

Disease	Biomarker name	Second-dimension analysis
Echinococcus granulosus	Protoscolex tegument paramyosin	2-DE and Western blot analysis
Potential biomarkers for *Helicobacter pylori*	CagI (25 kDa), urease G accessory protein (25 kDa), UreB (63 kDa), and pyrroline-5-carboxylate dehydrogenase (118 kDa)	OFFGEL electrophoresis, SDS-PAGE, Western blots, and MALDI TOF/TOF
Toxoplasma gondii	Microneme protein 10 (NcMIC10), phosphoglycerate mutase 1 (PGA), dense granule protein 7 (GRA7)	OFFGEL electrophoresis, SDS-PAGE, Western blots, and MALDI TOF/TOF
Acute *leptospirosis*	*Leptospira interrogans* protein	OFFGEL electrophoresis, SDS-PAGE, Western blots
Analysis of excretory-secretory antigen of *Entamoeba histolytica* for detection of amoebic liver abscess	*E. histolytica* lectin (152 kDa), *E. histolytica* pyruvate phosphate dikinase (110 kDa)	OFFGEL electrophoresis, SDS-PAGE, Western blots, and MALDI TOF/TOF
Detection of *lymphatic filariasis*	BmR1 with BmSXP	2-DE, Western blot, ELISA
Diagnosis of human *Toxocariasis*	TES-26, TES-30USM, and TES-120	2-DE
Serum proteome analysis of vivax malaria	Apolipoprotein A and E, serum amyloid A and P, haptoglobin, ceruloplasmin, and hemopexin	MALDI-TOF/TOF and mass spectrometry
Serum biomarkers to detect breast cancer	BC1, BC2, BC3, and CA 15.3	SELDI mass spectrometry LC-MS/MS
Malignant pleural effusion	Carcinoembryonic antigen (CEA)	2-DE, mass spectrometry
Peritoneal cancer dissemination	CEA	2-DE, mass spectrometry
Thyroid cancer metastasis	Thyroglobulin	2-DE, mass spectrometry
Pancreatic cancer	Carbohydrate antigen 19–9 (CA19.9)	2-DE, mass spectrometry
Lung cancer	CD98, fascin, sPIgR	1-D PAGE, nano-ESI-MS/MS, and ELISA
Potential biomarkers for osteosarcoma	Serum amyloid A (SAA), zinc finger protein 133 (ZNF133), tubulin-α1c (TUBA1C), gelsolin, peroxiredoxin 2 (PRDX2), cytochrome C1 (CYC-1)	2-DE, MALDI-TOF MS, Western Blot, and ELISA
Ovarian cancer	Leptin, prolactin, osteopontin, and IGF-II	Microarray analysis

et al. 2006). In most commercial diagnostic kits, a combination of antigenic extract is applied as it provides higher sensitivity and specificity than utilizing only one biomarker (Andersen and Espersen 1992; Glassman et al. 1990; Manes et al. 2005; Pelerito et al. 2006; Simor et al. 1996). Nevertheless, the designations of antigens applied in commercial diagnostic kits are commonly undisclosed.

Lateral flow dipstick tests (LFDs) are simple devices predesigned to discover the existence of antigenic proteins in a sample without the need for specialized and expensive apparatus; however, various lab-based approaches exist, which are supported by reading devices (Khalilpour et al. 2014b). A LFD test is designed to be composed of a conjugate pad, a nitrocellulose (NC) membrane with test and control lines, and an absorbent pad. The antigen is utilized as the test line by jetting it linearly onto a membrane card (Fig. 5a). The strip is comprised of an absorbent pad on top, and NC membrane in the middle, and sample pad at the bottom end (Fig. 5b). The LFD is placed in a well of a microtiter plate, and serum sample is enabled to flow up the dipstick via capillary function. Once the sample attained the uppermost of the strip, the dipstick is placed into alternative well comprising colloidal gold conjugated antihuman immunoglobulin G (IgG) antibody. After the line is well developed, the dipstick is descended in another well comprising chase buffer to rinse the excess colloidal gold-conjugated IgG. The outcome of experiment could be assessed within 15 min, according to the number of observed purplish red lines (control and test lines). Detection of one or two of these purplish red lines are recorded as "negative" and "positive," respectively (Fig. 5c, d) (Khalilpour et al. 2014b; Saidin et al. 2014). There are many reasons for a failing of the control programs of disease for instance large time gap between sample collection, analysis, and control application (Mamuti et al. 2002).

FAST-ELISA and dot-ELISA are various alterations of the ELISA that act as rapid tests, but the dipstick assay produces a more rapid and robust field applicable assay. Pappas et al. (1986) was the first who proposed the dipstick assay in 1986 as a potential devolvement of dot-ELISA. In this technique, nitrocellulose filter paper was divided into strips; marked by antigen or specific antibody; and then fixed to plane, pliable plastic strip via water-insoluble glue in order to oppose fracture (Pappas et al. 1986). Studies show that dipstick assays are not the same. Dot immunobinding assay (DIA) is another assay similar to the dipstick that combines the dot-blotting test

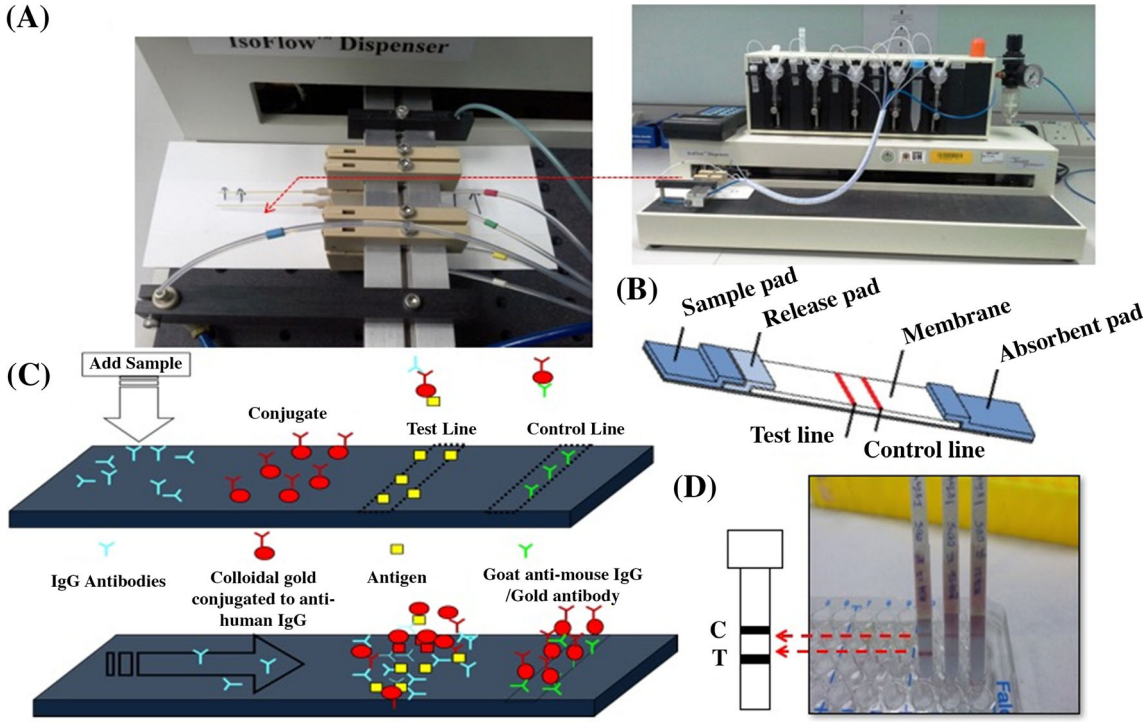


Fig. 5 Preparation and schematic of lateral flow dipstick. **a** Jetting it linearly onto a membrane card using IsoFlow™ Dispenser, **b** a lateral flow device in the dipstick format, **c** operation principle of a lateral flow dipstick, and **d** expected results of a lateral flow dipstick

and colloidal dye particle-linked antibodies, which was also introduced for the diagnosis of certain diseases (Olut et al. 2005).

Two other rapid immunodiagnostic tests, immunogold filtration assay (DIGFA) and immunogold-chromatographic lateral-flow assay (IGCLFA), are based on the colloidal gold conjugated antibody/antigen (Feng et al. 2010). Colloidal gold is a suspension or a colloid-like of submicrometer-size particles of gold in a fluid form. The color of liquid for particles less than 100 nm is usually intense red, and for larger particles, it is blue/purple (Wessling 1996). IGCLFA also known as immunogold chromatographic assay (IGCA) is a kind of dipstick based on an immunochromatographic procedure that gold conjugate and sample forms a complex, which migrates to the capture zone (antigen or antibody) on the nitrocellulose membrane through chromatography. Then, it binds to the solid phase via antigen-antibody interaction, accumulation of colloidal gold for detection confirmation, and reaction in precipitation as a line (Corp 1996). Therefore, rapid tests are independent of a reader device or costly apparatus, and they are simple, effective, and easy to use (Hujakka et al. 2003; Khalilpour et al. 2014b).

Microfluidic-based immunoassays and diagnostic tools

Microfluidic analytical systems, also known as "lab-on-a-chip" or "micrototal-analysis system" (μTAS), integrate all the miniaturized analytical stages performed in a laboratory (separation of a mixture, transportation of a fluid, chemical/biological reaction, and detection) and related compartments (valves, mixing chamber, sensors, etc.) on a single processor/chip (Fig. 6a). Microfluidic technologies are powerful methods, which facilitate the production of on-chip immunoassays for medical diagnostic devices and offer an alternative capable of replacing conventional methods. The advantages of these devices are on-chip detection and real-time monitoring of blood-related infectious diseases from a minor quantity of patient samples, detection facility, and in parallel multiple sample detection (El-Ali et al. 2006; Yakovleva et al. 2002). This technology could be used to incorporate different tests into a simple device with unique controlled reaction chambers. Microfluidic-based diagnostics of infectious diseases is potentially useful to produce fast and accurate results, increase sensitivity, and reduce volume of required sample (Parsa et al. 2008; Xiang et al. 2006). Thus far, nanofluidic/microfluidic devices have been applied for sample preparation. Examples include continuous fractionation of blood flow, purification of small proteins, and DNA/RNA extraction (Lee et al. 2010b; Lion et al. 2003). An example of microfluidic application in immune diagnosis is an on-chip diffusion immunoassay with capability of quantifying the concentration of minor molecules in the channels. This immunoassay employs antigen-specific antibodies in the microfluidic T-sensor. With this capability, it can characterize the dispensation of a labeled probe molecule when it distribute from one part to another (Lee et al. 2010b). Stokes et al. (2001a) demonstrated the detection of *Escherichia coli* using an antibody-based biochip via a

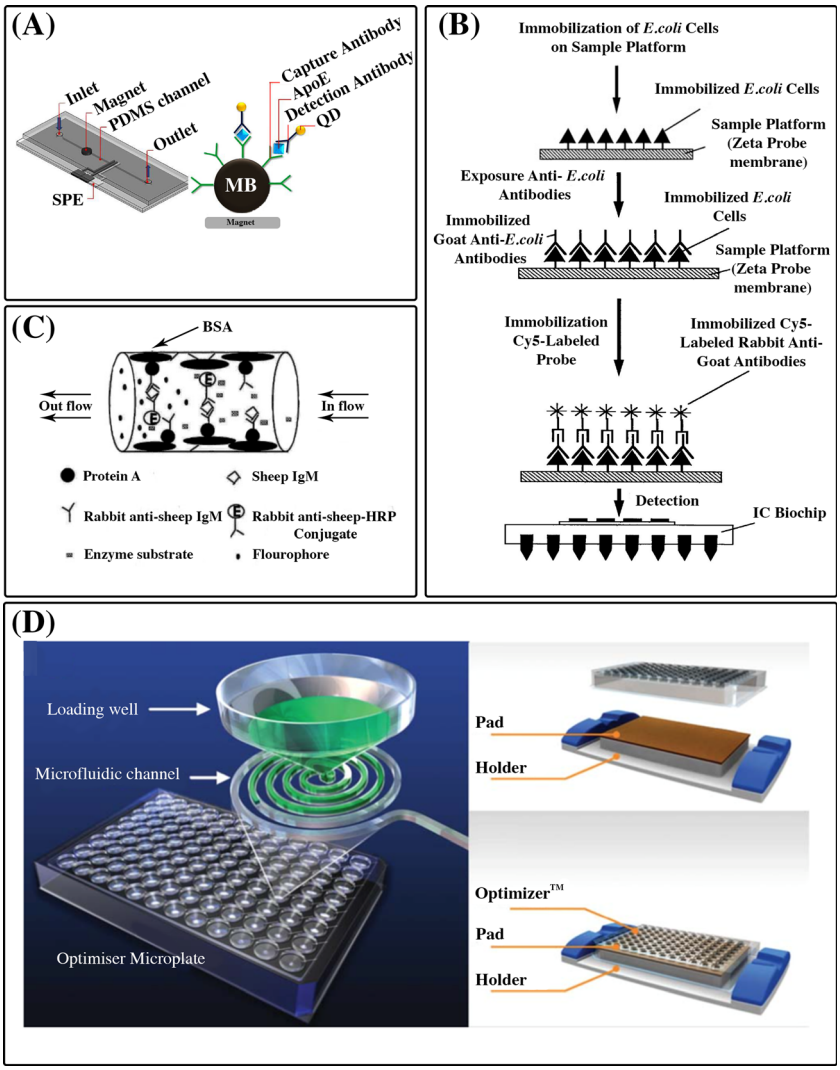


Fig. 6 Nano-fluidic/microfluidic technology. **a** Schematic of the lab-on-chip device components used for protein sensing, **b** schematic of the sandwich immunoassay for *E. coli*, **c** schematic of the ELISA assay in a microchannel, and **d** optimizer microplate illustration. Reprinted with permission from references ICN2 (2015), Eteshola and Leckband (2001), Stokes et al. (2001a), and Kai et al. (2012)

sandwich immunoassay. Their approach involves on-chip monitoring of bioassays by using Cy5-labeled antibody probes equipped with a microfluidic reagent delivery system (Fig. 6b).

An immunoassay determines the concentration of a specific analyte, namely, a specific antigen that is a point of interest in terms of biomedical research, i.e., warfare agents, foodborne pathogens, and disease pathogens (Ng et al. 2010). An immunossay can be classified either as homogeneous/heterogeneous or competitive/non-competitive. If the antibody is immobilized on a solid support, it is called heterogeneous, while if whole assay takes place in liquid phase, it is called homogeneous. The term competitive/non-competitive is used for the assays where the analyte inside a sample is competing with a labeled antigen for antibody binding (Ng et al. 2010). The performance of a microfluidic immunosensor strongly depends on surface modification, the way of introduction of sample to the device, immobilizing strategy of the antibody, and the sensing mechanism. Surface modification and microfluidic channels are generally a must since non-specific adsorption is a big issue in sensing that decreases sensitivity. For the purpose of surface modifications, various reagents like bovine serum albumin, glutaraldehyde, branched polyethyleneimine, linear polyethyleneimine, 3-aminopropyltrimethoxysilane, and 3-glycidoxypropyltrimethoxysilane have been used (Messina et al. 2008; Zhou et al. 2012). For introduction of antibody to a microfluidic device, a most effective strategy should be applied to successfully translate the laboratory practice to a microscale device. In general, pressure-, electrokinetic-, or capillary-driven flow is applied for liquid flow inside the microchannels. Immobilization of the antibody to the surface and/or channel walls is achieved either direct adsorption, covalent attachment, or microcontact printing (McDonald and Whitesides 2002).

Among other diverse and widespread applications, immunoassays hold great promises in μTAS. Lots of efforts have been made to overcome the drawbacks of ELISA by various microfluidic techniques coupling different sensing strategies, chemiluminescence, electrochemical, optical, etc. (Eteshola and Balberg 2004; Novo et al. 2011). Liu et al. (2009) designed a polymethyl methacrylate microfluidic chip coupled with electrochemical detection system to detect α-fetoprotein (AFP) based

Table 3 Lists some of the microfluidic-based immunosensor assays

Analyte	Assay type	Detection	Substrate	LOD	Analysis time	Flow
B-type natriuretic peptide	Heterogeneous	SPR angle shift	PDMS	100 ng/ml	30 min	Pressure
Staphylococcus enterotoxin B	Heterogeneous	Fluorescence	PDMS	0.5 ng ml^{-1}	Not mentioned	Pressure
Interleukin-6	Heterogeneous	Electrochemical	Plexiglas	0.41 pg/ml	25 min	Pressure
Escherichia coli	Heterogeneous	Fluorescence	Plexiglas	20 *E. coli*	<1 h	Pressure
Immunoglobulin G (IgG) and immunoglobulin M for dengue virus infection	Heterogeneous magnetic bead based	Fluorescence	PDMS	21 pg	30 min	Pressure
Tumor necrosis factor α	Heterogeneous	Fluorescence	PDMS	20 pg ml^{-1} (1.14 pM)	No data	Pressure
Insulin	Heterogeneous	Fluorescence	Glass	3 nM	30 min	Electrophoretic
17-β estradiol	Heterogeneous	Chemiluminescence	Poly(dimethylsiloxane)-glass hybrid	2.5 pg/ml	~20 min	Pressure
Human serum IgG antibodies	Heterogeneous	Electrochemical	Plexiglas	0.37 U ml^{-1}	25 min	Pressure
C-reactive protein	Heterogeneous	Fluorescence	PDMS	0.54 μg/ml	5 min	Pressure
hs-CRP	Heterogeneous	Chemiluminescence	Asymmetric polysulfone membrane and nitrocellulose membrane	1.05 ng ml^{-1}	~15 min	Delayed substrate release
Low abundant carbonylated proteins	Heterogeneous	Fluorescence	PDMS	Lesser than 700 ng protein	No data	Pressure
(CA125), HER2, epididymis protein (HE4), and eotaxin-1	Heterogeneous	Surface-enhanced Raman scattering	PDMS	15 fM, 17 fM, 21 fM, and 6.5 fM, respectively	No data	Pressure
EpCAM	Heterogeneous	Electrochemical	PDMS	2.7 pg ml^{-1}	10 min	Pressure
Immunoreactive trypsin	Heterogeneous	Laser-induced fluorescence	Glass	0.87 ng ml^{-1}	37 min	Pressure
H1N1	Heterogeneous	Fluorescence	Cyclic olefin copolymer	0–100 μg/ml	<3 h	Pressure
IgG	Heterogeneous	Electrochemical	PMMA	1 pg ml^{-1}	Not mentioned	Pressure
Carcinoembryonic antigen	Heterogeneous	Electrochemical	Paper	0.3 pg ml^{-1}	Not mentioned	Pressure
L. pneumophila	Heterogeneous	Surface plasmon resonance	PDMS	103 CFU/ml	Less than 60 min	Pressure
E. coli O157:H7	Heterogeneous magnetic bead based	Magnetoresistance	PDMS	105 colony forming units/ml	Not mentioned	Pressure
Bovine viral diarrhea virus	Heterogeneous	Light scattering	PDMS	103 TCID 50 ml^{-1}	Less than 5 min	Pressure

on enzymatic reaction of horseradish peroxidase (HRP)-conjugated antibody. Similarly, Novo et al. (2011) developed a lab-on-a-chip system where primary antibodies are adsorbed onto the microchannels made by polydimethylsiloxane (PDMS) via microspotting and detected by FITC- or HRP-labeled secondary antibodies using silicon photodiode in approximately 30 min with the linear range of nanometer to millimeter. Another study presented by Lai et al. (2004) shows the design of a combined microfluidic apparatus on a compact device and that carries out ELISA for the detection of IgG from hybridoma cell culture with a shorter assay time compared to conventional ELISA and less reagent consumption. Eteshola and Leckband (2001) have also developed an ELISA in PDMS microfluidic channel (Fig. 6c). In this assay, the microfluidic sensor chip was successfully used to quantify a model analyte (sheep IgM) with sensitivity down to 15 ng/ml (17 nM). The results demonstrated the feasibility of using plastic sensor chips for immunoassays. In the research by Kai et al. (2012), a modern "microfluidic microplate"-Optimiser™ microplate has been used, which has the ability to increase the levels of sensitivity. For this purpose using a ANSI/SBS-compatible 96-well plate, a microfluidic channel is designed for repetitive loading of sample at the bottom of every "well" (Fig. 6d).

Lab-on-chip or point-of-care (POC) diagnostics is another application of microfluidic technologies, where various assays can be integrated into a single device (Lee et al. 2010a). In the scope of POC diagnostics, microfluidic approaches address the drawbacks of minimization of expensive reagents, reducing the manufacturing cost and enabling mass production and miniaturization. Different groups have presented examples of usage of microfluidic platforms for food-borne pathogen detection (Ikeda et al. 2006; Stokes et al. 2001b) and infectious

diseases (Chen et al. 2007; Lee et al. 2010a; Lee et al. 2009). POC devices used for diagnosis of infectious diseases have several steps; first of all, an input like blood, saliva, or urine that contains analyte of interest is processed (separation, mixing/lysis, affinity/recognition, electrokinetic separation), then recognized/enriched, and finally analyzed (electrical, optical, colorimetric, mechanical) (Damhorst et al. 2015). Automated protocols for the rapid POC detection of some bacteria such as *Clostridium botulinum* toxin A, *Yersinia pestis*, *Staphylococcal* enterotoxin B, *Bacillus anthracis*, and *Bacillus subtilis* are accessible. According to manufacturing characteristics, restrictions of diagnosis are mostly 1–2 log units and up to 3 log units better than ELISA and lateral flow tests, respectively (Eteshola and Leckband 2001; Mairhofer et al. 2009; Stokes et al. 2001a). Table 3 lists some of the microfluidic-based immunosensor assays targeting various biomarkers. Majority of the POCs have been designed for the purpose of infectious diseases especially for HIV, malaria, and tuberculosis, the "big three," world's leading causes for mortality. The reference paper could be followed for the examples of POCs used for that purpose (Damhorst et al. 2015).

Conclusions

This review provides an overview of some of the tools necessary for effective protein marker analysis in medical diagnostic devices. Describing these technologies in depth goes beyond the scope of the present review. Indeed, at the rate that separations, mass spectrometry technology, bioinformatics, and diagnostic systems are constantly being improved and any chapter of this category is rapidly out of date. We also provided some insight on novel strategies for applications of microfluidic technology in proteomics and immunodiagnosis of some infectious diseases as a promising tool for addressing various limitations of the arena of proteomics. But there is still a need for assessment of their performances in terms of sensitivity, multiplexing, robustness, and applicability to real samples and integratability to current proteomic technologies. In a similar fashion, despite its exciting prospect for detection of infectious diseases, microfluidics is still an immature technology. With current technology, it is not possible to do real sample analysis on-chip with a simple microfluidic device that has self-calibration property, user-friendly interface, and flexible storage capacity that will enable to work under different ambient temperatures and long shelf life.

Considering that the efforts have been put up to now, it is expected that the current techniques for fluidic handling will be more versatile and multiplexed assays will be developed for multiple measurements of different analytes simultaneously. By miniaturization and improvements of devices like scanners and cameras used as sensing elements, telemedicine—the mobile health care seems to become more and more popular for POCs. Specially, paper-based microfluidic devices hold a great promise for diagnosis of infectious ailments in developing countries due to their capability of functioning in equipment-free fashion. However, new microfabrication processes that will enable mass production of bioactive paper substrates incorporating different biofunctional materials during fabrication should be developed. Many of the work discussed in the scope of this review will assist the transition from bench to market and help bridging the gap between research activities and commercialization efforts.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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