Review

Role of chromatographic techniques in proteomic analysis

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Abstract

Proteomics, the characterization of the proteome, is conceptually simple but technically challenging. Development of such technologies as mass spectrometry, multidimensional protein separation, and DNA sequencing has allowed the new field of proteomics to flourish. Proteomic analysis relies on a set of techniques chosen on the basis of the biological question. In any proteomic analysis, the first and most important task is the separation of a complex protein mixture, i.e. the proteome. Chromatography, one of the most powerful methods of separation, employs one or more inherent characteristics of a protein—its mass, isoelectric point, hydrophobicity or biospecificity. This review emphasizes high-performance liquid chromatography as an integrated part of technologies used to study the proteome, discusses the capabilities and limitations of current instruments, and highlights the potential of multidimensional liquid chromatography in proteomic analysis.
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Contents

1. Introduction

Chromatography has been used for centuries as a means of separation and, over time, has developed into a sophisticated analytical technique. It is a central technology in many fields of applied science such as the synthesis of drugs and pharmaceutics, purification of the products of organic synthesis, as well as food science, clinical chemistry and forensic science. Liquid chromatography has been used to separate organic molecules, DNA or peptides and proteins.

The success in analysis of DNA has lead to the sequencing of a number of genomes from archaea, prokaryotes, and eu-

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karyotes, including the human genome. Now that the Human Genome Project is nearing completion, the next demanding large scale project is the "Proteome". The proteome is the time- and cell-specific protein complement of the genome, encompassing all proteins expressed in a cell at any given time, including protein isoforms as well as co- and post-translational modified (PTM) forms. The study of the proteome is, compared to the genome, much more daunting for several reasons. While the genome of the cell is constant, nearly identical for all cells of an organ or organism, and consistent across a species, the proteome is extremely complex and dynamic as it continuously responds to such external factors as other cells, nutritional status, temperature, drug treatment, to name only a few. As a result, there is no fixed proteome. Therefore, any analysis of the proteome is a "snapshot." Moreover, study of a proteome is complicated by the dynamic range of protein expression within the proteome; it may vary by as much as 7–12 orders of magnitude compared to only five orders of magnitude for DNA [1–3].

The diversity and extent of proteome complexity will not and cannot be solved by a single technology. Proteomic studies over the last five years demonstrate that the most effective proteomic analysis of even the simplest biological system uses a combination of protein separation and identification techniques. To achieve the most comprehensive protein separation, old techniques have been refined and new ones introduced. As the result, the field of proteomics is evolving rapidly. In this review, we outline the role of chromatography, in particular liquid chromatography, as a means of protein separation in proteomic analysis.

2. Proteomics and its tools

Since the term "proteomics" was coined in 1995 by Wilkins and co-workers [4,5] as the study of a proteome expressed by a specific genome, the exploration of proteomics has been expanding with each new study. Early proteomic research concentrated on cataloging of proteins and development of protein databases; over 6000 scientific papers have made important contributions to this field [6]. To date, the proteomes of numerous prokaryotic and eukaryotic species have been analyzed [7–12], resulting in extensive protein databases of these organisms. With the sequence of human genome [13] it is possible now to undertake large-scale proteomic projects focused on human cells, organelles, specific tissues and organs.

The number of proteins expressed by the approximately 30,000 human genes can be up to 100 times greater due to the known diversity of mRNA processing as well as to post-translational modifications of proteins. More than 100 protein modifications are known and more will likely be discovered [14]. A full understanding of the molecular mechanisms involved in health and disease progression will require the identification of all forms of each protein involved in cellular processes. Proteomics has expanded to the point
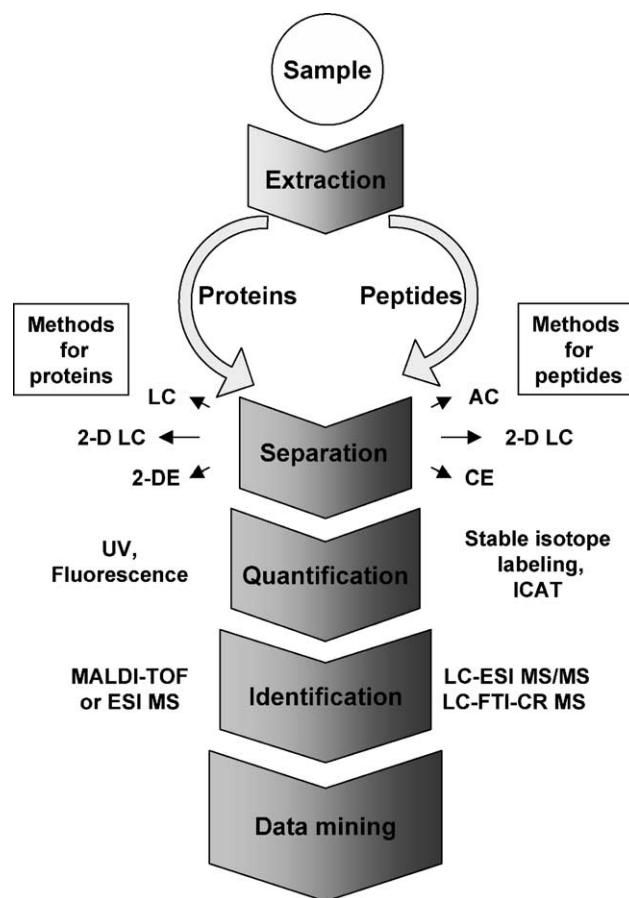

Fig. 1. Summary of technologies used at each step of proteomic analysis of intact proteins or complex mixture of peptides. Abbreviations: AC, affinity chromatography; LC, liquid chromatography; 2-D LC, two-dimensional liquid chromatography; 2-DE, two-dimensional gel electrophoresis; CE, capillary electrophoresis; ICAT^{TM}, isotope-coded affinity tag; MS, mass spectrometry; LC–ESI MS/MS, liquid chromatography–electrospray ionization tandem mass spectrometry; LC–FT-ICR, liquid chromatography–Fourier transform-ion cyclotron resonance mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry.

that it now incorporates the profiling of protein expression levels, identification of proteins and their modifications, characterization of protein interactions based on knowledge of their structure as well as the functional relations of individual proteins or all proteins in the context of the cell/organ/organism. Any protein related projects would benefit from the knowledge and expertise accumulated in differential expression, structural and functional proteomics.

The general strategy in proteomic research includes sample preparation, protein or peptide separation, their identification, and data interpretation (Fig. 1). Sample preparation is the first critical step that affects the outcome of the entire proteomic analysis. Several liquid chromatography techniques used to pre-fractionate samples are mentioned below. The next step is protein separation. For adequate representation of the proteome, only multidimensional separation techniques can provide resolving capability in thousands of protein species and have proven to be

superior to one-dimensional approaches. Until recently, two-dimensional gel electrophoresis (2-DE) was the technique most often used for protein separation. In the first dimension, proteins are focused according to their inherent charge, or isoelectric point (pI). Proteins are then resolved in the second dimension based on their relative molecular masses (M_r), typically by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The limitations of 2-DE in detecting low abundance proteins, very small or large proteins, as well as basic and membrane/hydrophobic proteins [15–17], as well as difficulties with automation of the process, have forced researchers to look for other methods of protein separation, such as liquid chromatography coupled to mass spectrometry (LC–MS).

An analyte delivered to a mass spectrometer has to be ionized either by electrospray (ESI) or matrix-assisted laser desorption ionization (MALDI) prior to its mass analysis. Analyte molecules can be of any mass and nature—small organic substances, oligonucleotides, polymers, peptides, or intact proteins. The accuracy of mass detection, which more precise for small rather than for large molecules, is an important factor for protein identification. Moreover, mass accuracy for intact proteins is over a range such that any modification that results in only a small change to the total mass of the protein will be within the error of the method. Therefore, modified and non-modified forms of the protein could not be distinguished. Since mass accuracy is best over the low mass range, analysis of peptides rather than intact proteins allows for superior mass detection and consequently, protein identification. For identification, proteins can be digested with endoprotease(s) either directly in the 2-D gel or in the liquid-phase before fractionation for MS analysis. Although digestion of the protein produces a complex mixture of peptides, it is beneficial due to increased overall solubility by elimination of non-soluble, most often extremely hydrophobic peptides. An assignment of detected peptides to the corresponding protein is achieved by comparing their masses to the theoretical masses of peptides obtained by in silico digest of the entire protein database. This method, based on the detection of peptides masses obtained following enzymatic digestion of a single protein or a mixture of proteins and known as peptide mass mapping or peptide mass fingerprinting (PMF) is a first step in protein identification.

More rigorous protein identification requires an amino acid sequence of at least few peptides. Analysis of these peptides via tandem MS (i.e. more than one mass analyzer, hence MS/MS) can reveal not only the amino acid sequence and, in some case, the sites of pos-translational modifications as well as previously unrecognized splice variants and protein isoforms. These features of MS/MS permit unambiguous protein identification. Both approaches, PMF and MS/MS, compare the resulting set of peptide masses and/or sequences of these peptides against those in existing databases. Accordingly, the availability of comprehensive protein databases based on the sequence of known proteins or deduced from genomic data is indispensable to the success of proteomic studies. In order to mine large data sets produced by multidimensional separation and identification techniques, high capacity computer systems and diverse bioinformatics approaches that allow rigorous qualitative and quantitative analysis are under active development.

3. Liquid chromatography as a method of protein separation

Liquid chromatography techniques (e.g., ion exchange, size exclusion, affinity, and reversed-phase), as well as electrophoretic separation in liquid-phase techniques (capillary isoelectrofocusing (IEF), capillary zone and free-flow electrophoresis (FFE)) are well known methods of protein separation and extensively described in the literature [18–24]. Each type of LC has already undergone numerous developments and improvements. The development of reversed-phase high performance liquid chromatography (RP-HPLC) will be used as an example to illustrate its progression and potentials for proteomic analysis.

Modern RP-HPLC utilizes a wide selection of chromatographic materials to separate proteins and peptides. The choice of packing material has the greatest impact on the separation and resolution of the proteins or peptides of interest. The separation efficiency of the packing material is determined by particle size, pore size, surface area, stationary phase, as well as the chemistry of the substrate surface. The most popular materials for RP-HPLC column packing are based on spherical silica. Typically, particle sizes of 3 and 5 μm are used for analytical separation of proteins, peptides or other small molecules. Separation efficiency increases by 30–40% when particle size is reduced from 5 to 3 μm for the same column length [25]. Particles that are smaller than 3 μm, which are both difficult to produce in uniform size and pack into a column, elevate backpressure beyond the pressure limit of most commercially available HPLC systems.

Unlimited access of the substrate to the surface of the packing material is achieved by increasing the available surface by means of expanded porosity of the particles. Depending on pore size, which can vary from 60 to 300 Å, over 90% of the available surface can lie within the pores. In order to generate a perfectly homogeneous surface, the quality of the silica has been improved so much that the highest purity type B silica particles are synthesized from metal-free reagents. To ensure the retention of the substrate by the silica-based particles, the surface is modified by alkyl chains varying in length from C_4 to C_{18}. The C_{18} bound phase is the most popular as it offers retention and selectivity for a wide range of compounds containing different polar and non-polar groups on their surface. C_4 and C_8 phases are used preferentially for separation of proteins, C_{18} for separation of peptides. There are also silica-based packing materials available for many different highly hydrophobic interactions, for example, acidic, basic, halogenated or phenolic [25] as well as ionic with minimal hydrophobic character [26].

An innovative approach to packing material for RP-HPLC is based on a novel surface-modified polymer-based separation media. By introducing two kinds of polymers—polymeric selectors into monosized polystyrene particles—Kaya and co-workers [27] were able to evaluate the responses of this packing material to such stimuli as temperature and pH. In that study, the stimulus-response behavior of the matrix was based on formation or disintegration of the complex between two polymeric selectors (polyacrylamide and poly(methacrylic acid)) due to changes in either temperature or pH. When the complex between polymers is formed, the property of the matrix is hydrophobic; as soon as the complex breaks down (at high pH or high temperature), each polymer is hydrated and the matrix becomes hydrophilic. This system employs water as a mobile phase and does not require organic solvents or modifiers that can change protein composition (i.e. eliminate existing PTM or, in contrast, artificially modify amino acids). Studies of the properties of this new material and modifications to how it is prepared demonstrate that it has potential for separation of biological molecules. Although this particular type of packing material has not been tested for its ability to separate biological molecules, another stimulus-responsive material, poly(*N*-isopropylacrylamide, PNIPAM) and its copolymers, have been used to separate steroids and organic acids under conditions of controlled temperature change during RP-HPLC [28,29]. A packing material with PNIPAM selector, in water, exhibited thermo-reversible conformational transfer from random coil to globule; hence, the surfaces of the particles changed from hydrophilic to hydrophobic, attenuating hydrophobic interaction between the stationary phase and solutes (Fig. 2). Improvements in separation will be achieved when an artificial material with appropriate stimulus-responsiveness that is specific for a given biological sample will be developed.

Porous packing materials work well for such relatively small substrates as small proteins and peptides obtained by proteolytic digestion of proteins. However, high molecular weight proteins or extensively modified proteins (for example, glycosylated proteins) have fairly large radii that cause peak broadening when using conventional porous particles. To minimize this,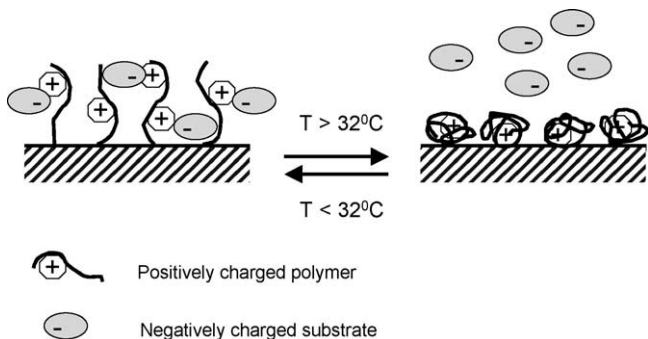 an array of macroporous, gigaporous, and gel-filled gigaporous packing materials has been introduced. Most recent materials are developed on base of nonporous particles such as grafted polymeric particles [30]. The cores of these particles are synthesized from a hydrophobic polymer which is then coated with a hydrophilic neutral polymer. Grafted chains, bearing groups with different functionalities, are then covalently attached to the polymeric hydrophilic surface. The resulting particles are over 10 μm and can have different shapes, providing a surface area sufficiently large for effective interaction with substrates. For example, cation exchange column packings based on pellicular polymeric particles grafted with ion exchange polymeric chains have proven to be extremely useful for high-resolution separation of a variety of differentially charged protein isoforms (Fig. 3). Future development of this type of stationary phase will undoubtedly benefit proteomic analysis.

Another aspect of renewed interest for RP-LC is the dependence of proteomics on the ability to resolve complex peptide mixtures for downstream MS analysis. Since the initial application of RP-LC for peptide separation in 1976 [31], numerous advances have increased peptide recovery, selectivity, and resolution through discovery of ion-paring agents,
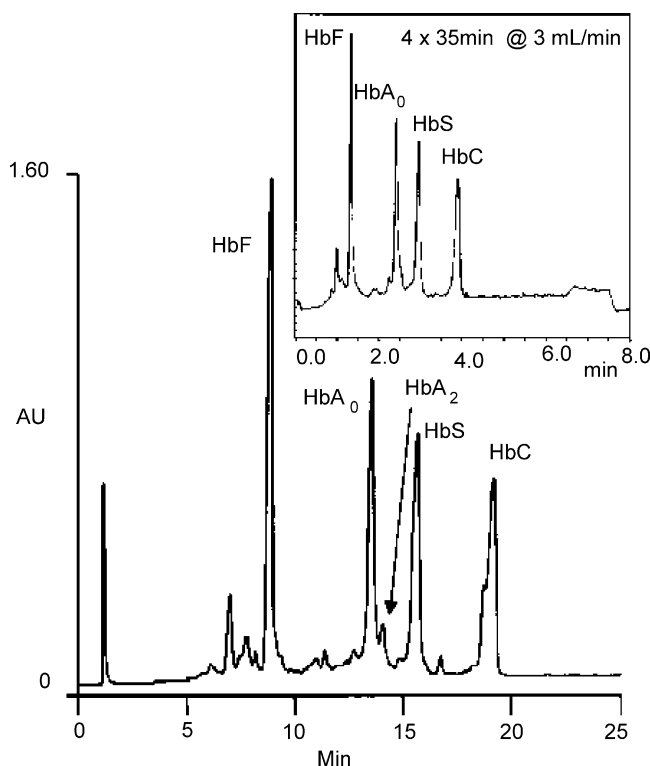
Fig. 3. Cation-exchange chromatographic separation of four haemoglobin variants including fetal, sickle cell, normal, and C haemoglobins. Column: 250 mm × 4 mm ProPac^{TM} SCX-10 cation exchanger. Eluents: (A) 10 mM sodium phosphate and 2 mM potassium cyanide at pH 6.0; B00.5 mM sodium chloride, 10 mM sodium phosphate and 2 mM potassium cyanide at pH 6.0. Gradients: at 1 mL/min 0–50% (B) in 30 min. Inset: fast separation of haemoglobin variants on short column (4 mm × 35 mm) at 3 mL/min flow rate. Reprinted from [30], with permission. Copyright release 2004 Wiley-VCH.

Fig. 2. Schematic representation of temperature-responsive chromatography.

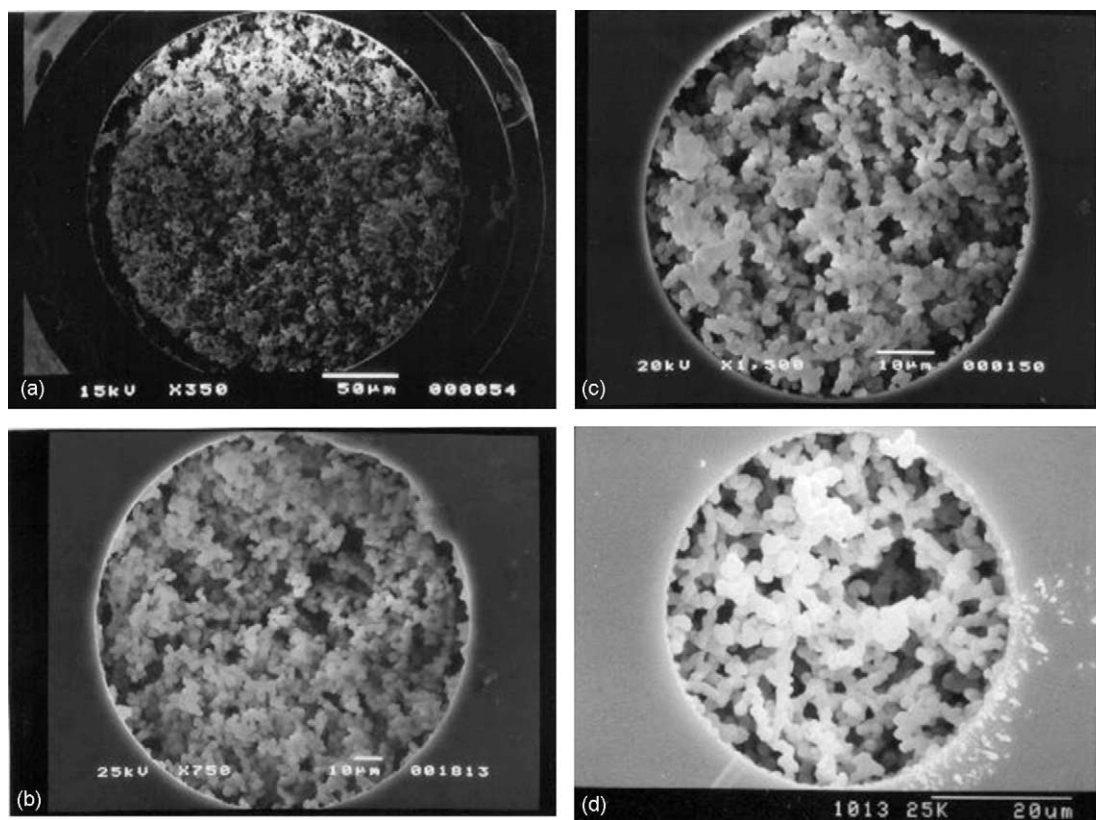Fig. 4. Scanning electron micrographs of monolithic silica in fused-silica capillaries prepared in (a) 250 μm, (b) 100 μm, (c) 75 μm, and (d) 50 μm capillary. Reprinted from [33], with permission.

high porosity of silica particles, and surface modification of packing materials. However, separation speed has not improved until recently. Modern mass spectrometers can now acquire spectra in a second and consequently can accommodate higher rates of delivery of analytes than current LC systems can produce. Hence, high-speed RP-LC packing materials are in demand. In porous materials, separation speed is limited by the rate of analyte transfer between the mobile phase and stationary liquid in the pores of the matrix particles. To enhance mass transfer, silica monolith columns were introduced [32–34]. These columns are comprised of continuous rod of silica-based gel which is made of highly interconnected network of large and small size pores created by a "sol–gel" process. The macropores (2 μm diameter) allow fast flow of the eluent while the fine pores (13 nm) offer the surface area required for the separation process (Fig. 4). Monolith material has a total porosity of over 80% that facilitates high permeability, good surface area, and enhanced mass transfer due to convection and not diffusion, resulting in practically no loss in peptide resolution, peak elution volume and concentration of analyte with flow rates ten times higher than conventional rates (10 ml/min versus 1 ml/min) (Fig. 5). According to Regnier and co-workers [35], separation of tryptically digested bovine cytochrome C on Chromolith C_{18} column had only one negative feature—carry over of analyte due to slow desorption of a small portion of the total analyte. The selection

of mobile or stationary phase can solve this problem. In general, decrease of runtime from 60 to 6 min for separation of an entire digest mixture or screening many new drugs is a very attractive feature for high throughput analysis in pharmaceutical development [36] and proteomics. By reducing the size of the columns to micro-size (capillary) and maintaining high flow rate of 1 ml/min, Tanaka and co-workers demonstrated that monolithic silica columns, under favorable conditions, performed better than conventional particle-packed columns [37]. This type of column can be easily adapted for LS–MS providing that the eluent stream is split for continuous MS analysis and collection of the analyte for later analysis. Thus, high-speed RP-LC will be of great value for peptide separation in proteomics.

The instrumentation that comprises a modern HPLC system has been improved and optimized to achieve accurate and reliable solvent delivery over wider flow rate ranges, to minimize the dead-space of the system, and to allow for efficient sample introduction through development of injectors and autosamplers. Traditional UV–vis photodiode array detection allows to obtain UV spectra of the separated compounds as they elute. However, one cannot differentiate by UV spectra alone if two or more proteins or peptides are co-eluting. Under these circumstances, only a mass detector can distinguish different compounds in the same fraction. As a result, new types of detectors—mass spectrometers—have
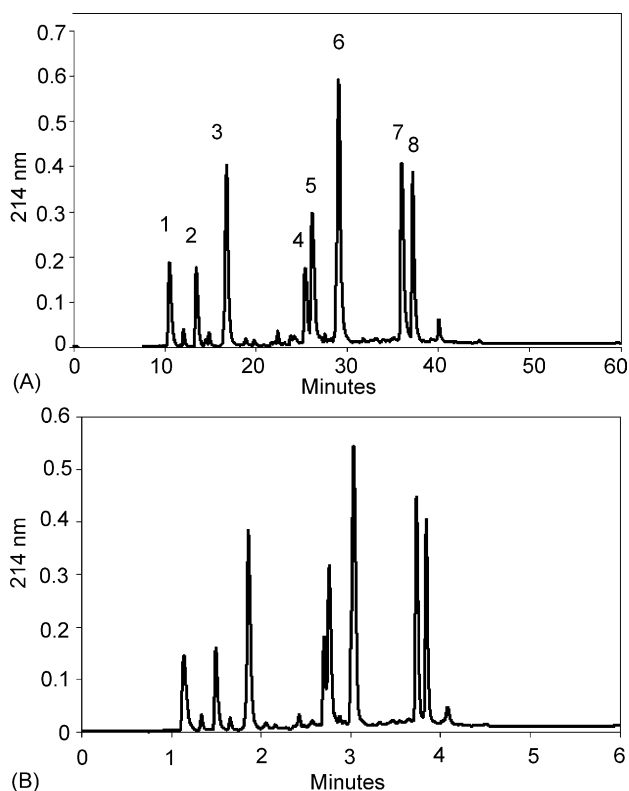
Fig. 5. Reversed-phase chromatograms of bovine cytochrome *c* tryptic digest at low flow rates of (A) 1.0 ml/min with 60 min gradient and (B) 10.0 ml/min with 6 min gradient. Mobile phase composition ranged from 5 to 60% acetonitrile in the presence of 0.1% TFA. Gradient volume was constant at 60 column volumes. Only moderate loss of separation efficiency was observed at high mobile phase velocity (A vs. B). Reprinted from [35], with permission.

become a preferred choice for the identification and characterization of biomolecules separated by LC. Over the last decade, LC–MS, as one of the most technically demanding systems, has been refined to such an extent that it is available to a broad range of research laboratories.

4. Developments in liquid chromatography for MS analysis

The most commonly used interfaces between LC system and the MS analyzer are matrix-assisted laser desorption ionization and electrospray ionization devices.

MALDI requires off-line sample deposition onto the target plate where it will be co-crystallized with the organic matrix suitable for excitation by laser to produce single charged ions. Even though MALDI-MS is considered to be a robust method of detection in the presence of salts and detergents, their levels have to be substantially lower than those resulting from routine biochemical manipulations. The most common application of LC in preparing a sample for MALDI is to 'clean' a peptide mixture obtained by tryptic digest. To remove salts and other contaminants, peptide mixtures

are passed through a RP microcolumn in a pipette tip (ZipTip). Elution of peptides from C_{18}-bound phase with high concentrations of organic solvent(s) allows one to purify and concentrate a sample. Affinity chromatography has been used successfully to enrich certain type of peptides: phosphopeptides with immobilized metal affinity chromatography (IMAC) microcolumns, biotinylated cysteinyl peptides captured with avidin, glycopeptides seized by glycans. Affinity and immunoaffinity treatment of the sample prior to MS analysis can be applied to whole proteins as well. This approach not only reduces the complexity of the protein mixture but also enriches low-abundance proteins. From a technical standpoint, these off-line sample preparations are well suited for further MALDI-MS analysis. Various examples of are described in reviews by Lee and Lee [23] and Issaq [38].

In ESI, a liquid flowing from a capillary in the presence of a high electric field causes charge separation during formation of a plume of droplets. ESI requires the delivery of substrates for ionization in the flow of a volatile and very pure solvent to ensure formation of true ions, not charged clusters or particles. The smaller the size of the droplet, the more rapid is the formation of ions at the higher charge density. Ideally, for 100% efficient ionization, the flow and concentration of the sample should be in the range that provides less than one analyte molecule per droplet. Conventional low flow columns have been replaced with microcolumns, hence microESI (μESI). The reduction of the column's internal diameter from 1 mm to 75 μm permits a decrease in flow rate from 50 to 0.3 μl/min [39]. These microcapillary columns are packed into fused-silica capillary tubing with outlet diameters as small as 8 μm and provide routine detection levels in the low-femtomole range for peptides. Typical microcolumns are packed with 1–3 μm diameter reversed-phase materials, most commonly C_{18} silica-based matrices. To eliminate leakage of packing material, sintered silica particles are used as frits. Preparation of columns with this type of frit is difficult and frits themselves can interact with peptides or proteins, causing peak broadening. Mann's group achieved high success rates of column preparation using the principle of stone bridge arch stability [40]. An "arch" of particles above the outlet formed at low pressure at the beginning of the packing process serves as a stable structure to retain the particles in the capillary but without clogging it. The idea of using self-assembled particles as the frit at the outlet of the tapered end column is an example of a technical improvement in LC that minimizes post-column dead volume, decreases peak width, and provides highly efficient peptide analysis for proteomics. It is also reflects the general trend of interfering as little as possible with the sample of the interest to ensure its comprehensive analysis.

EIS sources with flow rates in the microliter per minute range appear to be sensitive to the concentration of the sample. To increase sensitivity, nanoelectrospray (nanoESI) was introduced in 1994 [41]. By reducing the internal diameter of the capillary from 150 to 15 μm and, consequently, the flow rate to 20–40 ηl/min, the efficiency of ionization in-

creased ∼100-fold. This also meant that samples as small as 1 μl could be analyzed in ∼30 min. To achieve these conditions, high-efficiency narrow (15 μm) and long (up to 80 cm) columns equipped with a short pre-column of larger diameter provides a flow rate of 20 ηl/min at 10,000 psi and at the same time offer zeptomole detection limits for proteins [42]. With flow rates in this range, the challenge is to maintain high-efficiency separation that can process typical sample volumes and be effectively coupled to MS analyzer.

Capillary LC based on silica monolithic stationary phases has become more popular in the proteomic analysis of complex samples because it demonstrates excellent separation performance for peptides and proteins, is adjustable for low and high flows, is robust, contains easily regenerated packing material, and can be integrated in fully automated LC–MS systems. Due to the low backpressure, capillary columns can be up to 1 m long, allowing an increase in separation efficiency, especially for complex mixtures.

Given that the peptides and proteins separated by the LC technique of choice are delivered in a liquid-phase for their identification by one or more mass analyzers, LC–MS offers fast analysis of minute samples, flexibility for different LC separation methods, and automation for high throughput analysis. This makes the combination of LC and MS invaluable for large-scale proteomic projects directed toward human proteins, cells and tissues.

5. Multidimensional approach

Historically, biochemists have targeted a specific protein, and isolated and analyzed it using in vitro and/or in vivo assays and models. In the post-genomic era of biology—the era of proteomics—the goal is to monitor all protein interactions, all PTMs, and determine how these changes alter protein function as a result of upstream, downstream and/or parallel processes that occur in the system. The question arises: "Are we technologically equipped for this task?"

No single chromatographic or electrophoretic method (the main methods in protein separation) is capable of complete resolution of such a complex protein mixture as the proteome. However, by employing two or more orthogonal separation methods which are based on different mechanisms of separation, success is possible. This concept was initially suggested by J.C. Gidding, whose work on multi-component chromatograms led him to a more global view and the concept of multi-dimensional protein separation [43–46]. In his article titled "Concepts and comparisons in multidimensional separation," he states that "the term of multidimensional separation (should be viewed) in a broad context, which requires first that components be subjected to two or more largely independent separation displacements. However, a second criterion is imposed: the separation must be structured such that whenever two components are adequately resolved in any one displacement step, they generally remain resolved throughout the process" [43]. Publications at the start of the 1980's

showed the importance of high intrinsic resolving power of true two-dimensional separation. A paper chromatography performed in two dimensions is an example. In terms of peak capacity, this system has a resolving power of several hundreds [47,48]. Even though two-dimensional column systems to further enhance peak capacity were proposed at that time, they did not surpass other two-dimensional separation techniques. Interestingly, Gidding stated at the time, that "the 2-D electrophoretic techniques will provide the largest known peak capacity, numbering in thousands" [43]. He is still right. 2-DE has been used for protein separation since 1975 when Klose [49], O'Farrell [50] and Scheele [51] almost simultaneously published the methods based on isoelectric focusing in the first dimension and gel electrophoresis in the second. This method became dominant for the study of complex protein mixtures, especially with the possibility of identifying proteins from spots through advancements in MS. However, 2-DE is biased toward higher abundance proteins with intermediate pI and molecular weight. As a result, to enhance and compliment 2-DE, non-gel-based multidimensional approaches have been pursued.

In traditional 2-D LC, almost any combination of columns with different retention properties is available (Fig. 6). Since most current 2-D LC systems are interfaced with mass spectrometers, the choice for the first dimension separation has to be complemented by reversed-phase chromatography in the second dimension because, in this case, the samples, eluted from the RP column, are in the most desirable form for injection into the mass spectrometer. In this type of analysis, the most suitable candidates for the primary chromatographic separation include size-exclusion chromatography and ion-exchange chromatography. In 1997, Opiteck et al. [52,53] reported a 2-D system based on eight serially connected size-exclusion columns coupled to two parallel RP columns. By alternating two RP columns between loading and analyzing, they allowed all the effluent from the size-exclusion columns to be fractionated by RP chromatography (Fig. 7). Proteins in the final eluent were monitored by a UV detector. Plotting UV signal intensity during size-exclusion separation against retention time, the authors were able to produce a 2-D chromatogram that strongly resembled the format of a stained 2-D gel. Despite the fact that this system lacked the resolution capacity of 2-DE, it was useful for isolating over-expressed proteins of interest from bacterial cell lysates.

2-D LC analysis of a highly complex peptide mixture was demonstrated by Patterson and co-workers [54]. To separate peptides derived from digestion of conditioned media, a strong cation-exchange (SCX) column in combination with an analytical RP column was used. A C_{18} trapping column was placed between these two columns to concentrate analytes and to divert the flow of IEC buffer away from the RP column which was coupled to a mass spectrometer. The back-flushing of the trapping column into an analytical RP column using an organic solvent gradient allowed separation of the peptides and their subsequent analysis by tandem MS. This particular combination produced a 40% increase in the

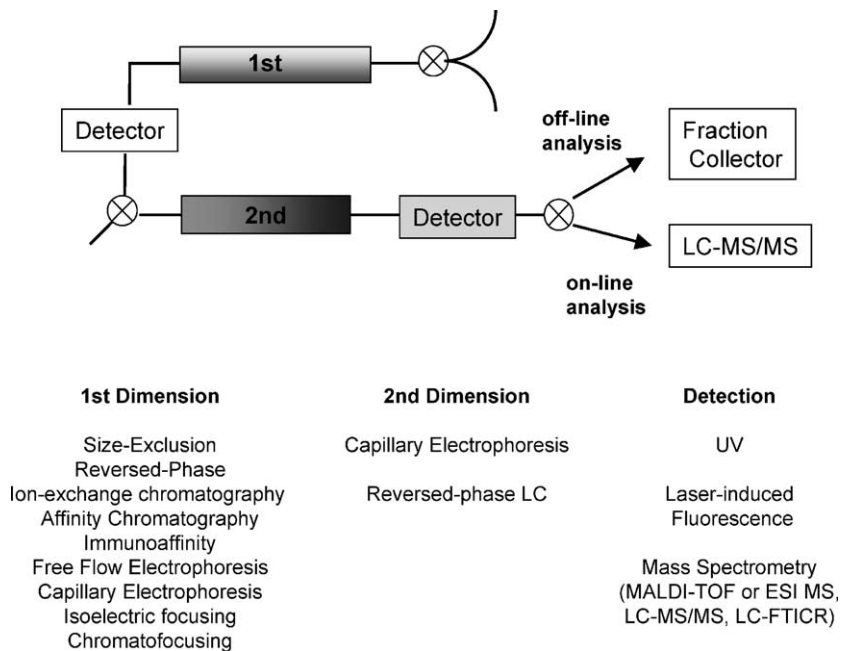| 1st Dimension	2nd Dimension	Detection
Size-Exclusion	Capillary Electrophoresis	UV
Reversed-Phase		
Ion-exchange chromatography	Reversed-phase LC	Laser-induced Fluorescence
Affinity Chromatography		
Immunoaffinity		
Free Flow Electrophoresis		Mass Spectrometry
Capillary Electrophoresis		(MALDI-TOF or ESI MS,
Isoelectric focusing		LC-MS/MS, LC-FTICR)
Chromatofocusing		

Fig. 6. Schematic representation of 2-D LC system for an analysis of complex protein mixtures and a table of methods and technologies used for each step of the analysis.

number of peptides and identified proteins compared to an un-fractionated control. In example of 2-D LC separation shown in Fig. 8, tryptic peptides obtained from proteolysis of whole cell yeast extract were also separated by SCX chromatography in first dimension and RP LC in second dimension. More than 12,000 unique peptides were sequenced and more than 1600 unique proteins were identified from a single sample [39]. This approach allows a large-scale screening of samples
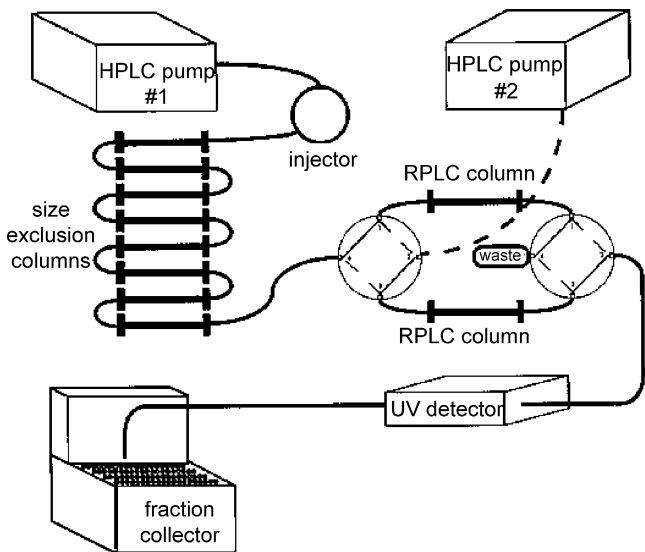

Fig. 7. Schematic representation of 2-D LC/LC/MS system showing the 8 SEC columns arranged in series for an effective column length of 2.4 m, the parallel 33 mm-long PR-LC columns in the LC/LC interface, and the location of the UV detector prior to fraction collection. Reprinted from [52], with permission.
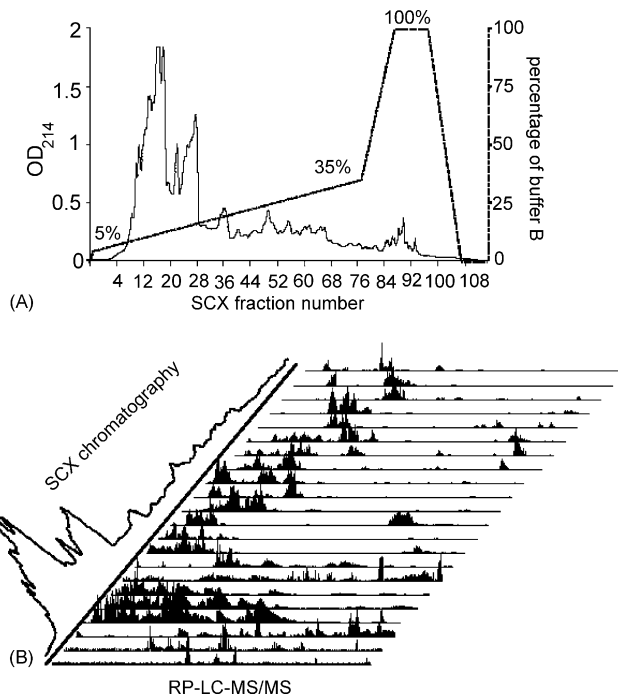

Fig. 8. Multi-dimensional peptide chromatography permits the analysis of thousands of proteins from a single sample. In the example shown, 1 mg of whole cell yeast extract was proteolysed with trypsin under reducing conditions. (A) The highly complex peptide solution was separated in the first dimension by strong cation-exchange chromatography with UV detection and fraction collection every minute (solvent A, 5 mM phosphate buffer, 25% acetonitrile, pH 3.0; solvent B, the same as A with 350 mM KCl). (B) The collected fractions were then analyzed individually by the nanoscale microcapillary LC/MS/MS techniques. Reprinted from [39], with permission. Copyright release 2004 John Wiley & Sons Ltd.

for protein identification. For a more detailed description of the various coupled 2-D LC systems, we recommend reviews by Liu et al. [55] and by Wang and Hanash [56].

One technical limitation to a multidimensional LC system is the requirement of at least two divert valves and pumps. As such, more complex 2-D LC systems suffer from a lack of automation due to the need for multiple sample loops, splits and switching valves used to transfer samples between columns and to correct flow rates. Moreover, to achieve true orthogonal separation in 2-D LC system, the resolution obtained in the first dimension step has to persist through the second dimension. Resolution is also affected by manipulations of the eluate flow between the columns. This problem was solved in Yates's laboratory with the creation of the biphasic column [57]. The merging of the different stationary phases into a single column eliminated the multiple manipulations and made the system amenable to automation. A biphasic column is a capillary (100 μm internal diameter) packed with strong SCX resin followed by RP resin. The proteins loaded on a biphasic column are separated in the first dimension by charge (SCE chromatography) and in the second dimension by hydrophobicity (RP). The process involves increasing salt concentration elutions, alternated with a ramp of organic solvent to elute analytes to the RP resin. Further separation on this resin is accomplished by a gradient of organic solvent. Analytes, eluted from the RP matrix, are directed into the ESI interface of a tandem mass spectrometer. Thus, the protein separation is achieved orthogonally and in automated mode. This unique biphasic column-based technique provided the foundation for a process called multidimensional protein identification technology (MudPIT). This technology, to date, is the most successful at global analysis of complex protein mixtures.

In the past, various technologies have shown promise as alternatives to 2-DE in terms of resolution and high-throughput in proteomics. With the emergence of the combination of nanoflow high-performance capillary LC–MS, attention is now being refocused on utilization of multi-dimensional liquid-phase based separation of proteins.

6. Applications of chromatography as a part of proteomic analysis

Chromatography is the most versatile technique available for protein and peptide separation and can be used for single analyte purification or profiling and analysis of complex protein and peptide mixtures. In this section, we provide a few examples of how LC can be utilized according to the complexity of a biological system.

6.1. Liquid chromatography for small molecules

The task may be as simple as purification of peptides, hormones or small proteins as pharmaceuticals. For example, the synthetic peptide fragment of human interleukin-β must be absolutely pure in order to minimize any side effects on the immune system. Efficient purification of small to multi-gram amounts of this crude synthetic peptide is achieved through 2-D LC using a combination of RP and ion-exchange displacement chromatography [58]. Multidimensional LC is an essential analytical and preparative chromatographic tool in the pharmaceutical industry. The need for increasing high throughput screening of biological fluids such as plasma, urine, saliva or bile for drug tracking, metabolites and other small molecules has driven the development of sequential and parallel HPLC systems with various combinations of RP, solid-phase extraction (SPE), restricted access media (RAM) SPE, and immunoaffinity columns [59]. Along with high resolution and sensitivity, multidimensional LC systems offer adequate high throughput and reproducibility and are easily coupled to detection systems such as MS and NMR.

6.2. Liquid chromatography in clinical proteomics

Using proteomic approaches and technologies to search for biomarkers of a disease for diagnosis, prognosis and risk stratification, as well as for monitoring the effectiveness of treatment is termed clinical proteomics [60]. The quest for small molecules, peptides, or proteins secreted into body fluids (particularly plasma or serum) represents the next level of separation complexity over purification of a single analyte. In this situation, the protein(s) of interest must be isolated from plasma containing many proteins with a huge dynamic range of concentrations [61]. The most direct approach in this case is affinity chromatography due to its extreme selectivity based on the molecular recognition between ligand and target. Removal of high abundance proteins (including albumin and IgG) is the key strategy in multi-component immunoaffinity-based protein subtraction chromatography (IASC), introduced by Anderson and co-workers specifically for analysis of plasma [62]. This method facilitates the search for low abundance biomarkers by removal of the dozen most abundant proteins from plasma.

Affinity chromatography is a powerful tool for study of protein–protein interactions. Most common affinity ligands are peptides, oligonucleotides (RNA or DNA fragments) and antibodies that can be generated by high-throughput combinatorial biochemistry [63], although aptamers, lectins, biotin, and metals are also widely used. Furthermore, there are numerous examples of affinity-based separation of proteins, glycoproteins, peptides containing cysteine, tryptophan or metheonine, histidine-tagged proteins and peptides, and phosphopeptides purified using metal-containing resins [64,65].

Recent advancements in surface-enhanced laser desorption/ionization (SELDI), which involves retaining proteins on a solid-phase chromatographic surface (ProteinChip® array) and direct detection of the retained proteins by time-of-flight (TOF) MS, is an example of "proteomics on a chip" [66,67]. Although not a classical form of chromatography, it does involve selection of proteins or peptides based on their

chemical properties prior to analysis by MALDI. SELDI is advantageous for rapid screening of minute samples. For example, body fluids obtained from patients can be analyzed for differences in profiles [68]. Unfortunately, the level of detection still remains in the low picomole range and the ability to identify proteins from SELDI is still challenging [69], unless affinity chromatography is used as a selection criteria. Whether appropriate specificity can be achieved by protein/peptide profiling without identification of candidate biomarkers is not clear [70] until further validation is carried out on large unbiased cohorts.

6.3. Complimentary approaches—expanding the view of the proteome

Analysis of the entire proteome of a cell, organ or organism is difficult if not impossible, particularly in higher order eukaryotes such as man. The most common approach today is to divide the cell into parts or subproteomes, thereby simplifying protein separation and identification [71]. Even so, subproteomes such as mitochondria, nucleus, cytoplasmic/soluble cellular pools can have 1000's of proteins. In this case, multidimensional protein separation is required. For example, cytosolic proteins from cancer cell line have been identified by Lubman and co-workers who have used 2-D LC systems comprised of an isoelectric focusing step in the first dimension and a nonporous RP separation in the second, followed by MS [72]. Proteins in the liquid-phase were separated by isoelectric focusing into 20 fractions. Each fraction was then subjected to separation on a nonporous C_{18}-coated silica-based RP column. This new packing material improves protein recovery, speed of separation, and resolution. The eluate from RP was split between direct on-line analysis of whole mass proteins by ESI–MS and off-line PMF by MALDI-MS of enzymatically digested fractions. Since many of the proteins in these human cancer samples were modified compared to their known masses in the database, only the combination of accurate intact mass, pI and peptide mass mapping can produce a protein map of this cell line. This 2-D LC approach improved the resolution of low molecular mass and basic proteins compared to 2-DE of the same sample [73].

Hoffman et al. [74] published an interesting study on cytosolic proteins-enriched extract obtained from a human colon carcinoma cell line where proteins were separated by free flow electrophoresis in the first dimension. The principal of FFE is based on continuous injection of sample into a carrier ampholine solution flowing as a thin film between two parallel plates. By introducing an electrical field perpendicular to the direction of flow, proteins are separated by IEF according to their pI and finally collected through a 96 channel tubing outlet. The advantages of this method are improved sample recovery due to absence of solid membrane supports and an increased range of sample loading capacity varying from analytical to preparative. By performing the IEF step under non-denaturing conditions, this technique provides an opportunity for studying protein complexes such as those oc-

curring in cellular signaling. In addition to its use as protein separation tool, FFE has also been used to separate cellular organelles [74,75]. To date, this technique is not readily interfaced with MS. However, in the future, a combination of FFE and RP chromatography could be made compatible with MS.

The multidimensional separation achieved by biphasic column chromatography in combination with MS, used in the MudPIT approach, has led to the identification of 1484 proteins in a soluble part of *S. cerevisiae* cell lysate, including low-abundance proteins, proteins with extreme p*I* values, and proteins with transmembrane domains [76]. In a typical MudPIT experiment, the entire protein sample is subjected to proteolytic digestion prior to separation. Trypsin is the common choice for the digest because it cleaves specifically at lysine or arginine—two highly abundant amino acids found throughout the sequences of most proteins. There are several reasons for identification of proteins at the peptide level: (i) to increase the overall solubility of the sample; (ii) accurate masses of intact tryptic peptides can be used for unambiguous protein identification; and (iii) sequencing of peptides via tandem MS may reveal the sites of PTM, previously unrecognized introns, or splice variants and protein isoforms. The digestion produces an even more complex mixture of peptides than in the original protein sample. As a result, thousands of peptides can be detected in each fraction submitted for MS analysis, including the unique subset of peptides separated by the biphasic chromatography. The prime benefit of MudPIT is that it facilitates high-throughput protein identification of an extremely complex mixture by combining the resolving power of high-performance chromatography with the analytical capacity of tandem mass spectrometry and bioinformatics. At the same time, the high-throughput of this technology is limited by the rate at which the MS can switch between MS and MS/MS modes, thus "undersampling" eluting peptides. In a typical experiment, only about 10–20% of tandem mass spectra lead with reasonable confidence to the identification of unique peptides [39]. In addition, the tens of thousands of mass spectra produced in a single experiment demand extremely high processing capacity and bioinformatics support.

A new strategy for global protein analysis was recently introduced; it uses a combination of high-efficiency capillary LC and high-performance Fourier transform-ion cyclotron resonance (FT-ICR) mass spectrometers coupled through an electrospray interface. Exploration of various applications of LC in combination with FT-ICR for characterization of complex protein mixtures, detection of PTMs and quantification efforts are underway in the Environmental Molecular Sciences Laboratory at Pacific Northwest National Laboratory (Richland, WA, USA) led by R.D. Smith and the National High Magnetic Field Laboratory at Florida State University (Tallahassee, FL, USA) led by A.G. Marshall.

FT-ICR MS offers an unrivaled mass resolving power ($m/\Delta m > 10^5$ for 5–10 kDa species), sensitivity (attomoles to zeptomoles), and mass measurements accuracy (<1 ppm) [42,77,78]. Thus, it is possible to determine the mass of a

peptide so accurately that it is unique among all possible peptides predicted from a genome. LC–FT-ICR analysis has been performed on cytosolic tryptic peptides from *S. serevisiae*, yielding >9000 peptides corresponding to approximately 1000 proteins predicted from unique yeast open reading frames [77]. This method, known as accurate mass and time (AMT) tags, determines accurate mass and elution time for each peptide predicted from genome. These AMT tags serve as confident markers to identify peptides in subsequent samples without sequencing each peptide and result in much greater throughput. Dynamic range enhancement applied to mass spectrometry (DREAMS) technology employed during a LC–FT-ICR run provides an accumulation of low abundance species and their analysis, resulting in a 40% increase in the number of identified putative peptides from yeast tryptic digest [79].

It is important to note that all of the global approaches described, recently termed "shotgun proteomics", have only been applied to reasonably simple organisms. For experiments designed to identify as many components as possible, too high a level of complexity will result in a less than complete survey of peptides in the mixture owning to the duty-cycle time of the MS to perform sequences of only chosen peptides. The alternative approach is to devote time to the separation step prior to mass spectrometric analysis via extensive (sub) fractionation in order to detect all components present and fully utilize the dynamic range of the system. The lack of complete and accurate genome sequences for higher species will become more critical as the analysis of low abundance proteins increases and unique proteins are more frequently encountered. Therefore, techniques for enrichment of specific subpopulations of the proteins and peptides will be very desirable.

6.4. Quantitative approaches

The significance of quantitative changes of any form of a protein in a cell is tremendous, given the highly non-linear relation between the amount of these changes and their biological consequences. From this point of view, quantitative protein profiling is an important part in the proteomic analysis of healthy versus diseased states. Protein quantification by differential staining on 2-DE gels is a popular approach but can be misleading since multiple proteins can be present in one spot on a gel. Thus, tracking the protein responsible for differential staining is difficult. To circumvent this problem, changes in protein expression can be measured directly from peptides. Recently developed methods for pair-wise protein quantification have employed stable isotope labeling [80–83]. In 1999, Gygi et al. [80] introduced the isotope-coded affinity tags (ICAT) method based on a three-dimensional chromatography (cation exchange, biotin affinity and reversed-phase) of the peptides generated by enzymatic digestion of tagged proteins. The ICAT reagent consists of a thiol-reactive group that specifically binds to cysteine, a polyether linker synthesized in an isotopically normal or heavy form where deuteriums replace hydrogens, and biotin affinity tag allowing tagged peptides to be purified [84]. Two protein mixtures are labeled separately with heavy versus light ICAT reagents, then combined and subjected to proteolysis with trypsin. The ICAT reagent-labeled peptides of the same sequence from the two samples are chemically identical and behave similarly in the subsequent chromatographic separation. Typically, RP LC–ESI–MS/MS is performed to identify proteins and sequence peptides. The change in abundance of each protein between the two samples is calculated by comparing the areas under the curve in the elution profile for each of the two peptides having identical sequences but different masses due to isotopic substitution in ICAT reagent. Since the entire quantification relies on the chromatographic elution profile, the optimization of RP LC for high-resolution of peptides to obtain high accuracy and dynamic range for peptide quantification is essential and under constant scrutiny [85]. For global quantitative comparisons of complex protein mixtures, the ICAT method was applied to the quantification of 491 membrane proteins from microsomal fractions of non-differentiated and in vitro differentiated human myeloid leukemia cells. This is the largest quantitative proteomic analysis to date [86]. The development of less expensive and more efficient methods based on differential chemical derivatization of the samples prior to separation and MS analysis has also been reported [87–89]. A multidimensional approach has also proven to be useful in these cases.

Nowhere in proteomics are the challenge so daunting and the technology still so limited for defining the chemical identity and functions of modified protein forms, or PTMs. For example, phosphorylation is one of the most common PTM. The low stoichiometry of this modification and its concomitant occurrence at multiple sites constitute an analytical and technical challenge. Glycosylation—another common modification, responsible for cell–cell communication and recognition, presents a challenge not only for identification of the site but also determination of the glycan structure. Characterization of PTM remains relatively unexplored in modern proteomics, partly due to the lack of sensitive and sufficiently robust methodologies. Nevertheless, there is little doubt that, as with quantitative proteomics, multidimensional approaches will eventually permit identification of various PTM [90].

7. Conclusions and future perspectives

Effective peptides and proteins separation, quantification and identification have become the central topic in proteomics. Liquid chromatography in particular has undergone extensive improvements and adaptations to become a multidimensional technique of proteins and peptides separation. Advances in multidimensional chromatography along with mass spectrometry will allow high-throughput analysis of minuscule samples. Indeed, there is already high demand for miniaturization of proteome analysis techniques. Microfab-

ricated fluidic devices that combine micellar electrokinetic chromatography and high-speed open-channel electrophoresis on a single structure for rapid automated two-dimensional analysis of peptides has been used for the analysis of peptide mixtures with peak capacity in the 500–1000 components range [91]. Microfluidic systems coupled to ESI mass spectrometer offer multifunctionality, reduced sample consumption, reduced dead-space, and increased MS detection sensitivity by 1–2 orders of magnitude. According to Lion et al. [92], microfluidic devices coupled to ESI–MS have been used for immunoassay, one and two-dimensional analyte separation and fulfill the requirements for proteomics. Development of microfluidic chips will be the basis for a leap into nano-scale proteomics.

As the field of proteomic matures its application to biology is expanding greatly. The information gleamed from proteomic studies will have greater direct impact on the quality of human life. For example, plant proteomics is accelerating due to the increased availability of genomic sequences for plant species, particularly important crop plants [93]. Identification of allergens (most of them are proteins, glycoproteins or peptides) will also benefit from technical development of high-performance chromatography integrated into the arsenal of proteomic tools [94]. Due to the importance of proteins responsible for cellular aging, dementia, and geriatric diseases another area in which proteomics will be appreciated is gerontology [95,96]. An application of LC-based technology is also valuable for proteomic studies of pathogens and the expression of virulent proteins because the molecular interactions of microbes with healthy host cells are mediated by membrane proteins. The completion of the genome sequences of several pathogens presents opportunities to develop new vaccines in a more rational manner, unlike most current vaccines that have been developed empirically. The discovery and development of these new vaccines are likely to be accomplished through integrated proteomic strategies [97,98]. Certainly, chromatography will have a central role along side other separation techniques in the application of proteomics in the future.

Acknowledgements

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