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Review

Sample preparation and fractionation for proteome analysis and cancer biomarker discovery by mass spectrometry

Sample preparation and fractionation technologies are one of the most crucial processes in proteomic analysis and biomarker discovery in solubilized samples. Chromatographic or electrophoretic proteomic technologies are also available for separation of cellular protein components. There are, however, considerable limitations in currently available proteomic technologies as none of them allows for the analysis of the entire proteome in a simple step because of the large number of peptides, and because of the wide concentration dynamic range of the proteome in clinical blood samples. The results of any undertaken experiment depend on the condition of the starting material. Therefore, proper experimental design and pertinent sample preparation is essential to obtain meaningful results, particularly in comparative clinical proteomics in which one is looking for minor differences between experimental (diseased) and control (nondiseased) samples. This review discusses problems associated with general and specialized strategies of sample preparation and fractionation, dealing with samples that are solution or suspension, in a frozen tissue state, or formalin-preserved tissue archival samples, and illustrates how sample processing might influence detection with mass spectrometric techniques. Strategies that dramatically improve the potential for cancer biomarker discovery in minimally invasive, blood-collected human samples are also presented.

Keywords: Antibodies / Diagnoses / Plasma / Serum / Urine

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1 Introduction

Proteomics incorporates the study of expression patterns, molecular interactions and functional states of proteins present in a cell, organ, or an organism under consideration. Although DNA may provide the blueprint for cellular functions and development, it is proteins that are the bricks and mortar from which life is built, and they are the molecules that perform the functions and dynamic processes that ultimately determine the phenotype of the cell. Cellular messenger ribonucleic acid (mRNA) expression levels may not necessarily be cor-

related directly to protein expression levels; therefore, monitoring the protein changes of cells is a more meaningful measurement parameter of cellular homeostasis. Minor changes in protein expression may be responsible for altering protein cascades, which may eventually lead to transformation and uncontrolled growth of cells. Furthermore, mutations may lead to processes that allow the cell to become tumorigenic and ultimately metastatic. Since proteins are the agents that control these processes, it is important to understand the changes in global protein expression patterns in order to be able to identify proteomic biomarkers that are involved in various cellular functions, differentiation, regulation, proliferation, and cancer progression [1].

The availability of global profiling strategies combining genomic sequencing with powerful high-throughput (HT) screening methods that provide high resolution has facilitated the comprehensive quantitative identification, relative abundance levels, and post-translational modification (PTM) states of proteins across various tissues and cellular fractions in a systemic manner [2]. However, the understanding of the diverse structural charac-

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Abbreviations: Abs, antibodies; FFPE, formalin-fixed and paraffin-embedded; HT, high-throughput; LAP, low abundant protein; mRNA, messenger ribonucleic acid; PCT, pressure cycling technology; PM, plasma membranes; PTM, post-translational modification; SELDI, surface-enhanced laser desorption ionization

teristics and interactions of the dynamic proteins represent a significantly greater analytical challenge than that posed by the static nucleic acid analysis required for deciphering the genome sequence. Elucidation of large-scale perturbations resulting from pathological processes to proteomic patterns provides insight as to the reason of such changes, as well as offering the potential of discovery of clinically relevant proteomic biomarkers of disease states that have substantial diagnostic, prognostic and/or therapeutic potential or disease recurrence, as well as facilitating risk assessment modalities [3].

The best strategy for sample preparation would be no sample preparation at all; however, the complexity of the dynamic proteome far exceeds the capacity of the currently available analytical systems. In higher animals, and plants, proteomic methods employing mass spectrometers do not presently – nor expected in the immediate future – define more than a small portion of a proteome on a routine HT basis. This limitation precludes analysis in a simple one-step process of the whole proteome of any complicated eukaryotic organism. Therefore, all proteomic methods are *a priori* targeted for simplification by fractionation and separation steps in some way either intentionally, or by the limitation of the analytical method employed to explore specific structural functions of interest using either chromatography, or other methods. Lacking a better alternative, therefore, this current prevailing approach simplifies the proteome while preserving most of the vital information essential for a meaningful analysis by today's methods [4].

It is generally assumed that in order to provide adequate analysis, the analytical method needs to include the following steps: (a) sampling, in which the sample is a good statistical representation of the investigated population; (b) specimen preservation, during which the sample is expected to be kept stable until the analysis is completed; (c) appropriate sample preparation; and (d) statistical analysis and bioinformatics data treatment. A major bottleneck of this analytical process lies in the sample preparation step, as it is often a time-consuming, and a laborious step. The purpose of any sample preparation technique is the cleanup of the sample and/or the extraction, enrichment or preconcentration of the analytes in order to improve the quality of the analysis. However, it is important to consider that any sample treatment depends on both the nature of the sample as well as the analytical technique that will follow, which in case of current proteomics will always require a case-by-case development, as no universal preparation technique is currently applicable to all proteomic samples [5].

Ideally, sample preparation should be as simple as possible in order to reduce time and avoid introduction of steps that could lead to sample loss, while eliminating interferences from the matrix (tissue). Moreover, it

should preferably include, if needed, a dilution or a concentration step in order to obtain an analyte at a concentration that is optimal for further MS analysis. Furthermore, it may be necessary, sometimes, to transform the analyte into a different chemical form in order to facilitate either the separation or detection of the protein under consideration [5].

2 Strategies for sample preparation

2.1 Approaches relying on physicochemical characteristics

Approaches relying on physicochemical characteristics for sample purification and enrichment prior to protein analysis are widespread as discussed below.

2.1.1 Tissue disruption and cell lysis by homogenization

Homogenization is one of the preparation steps employed for preparation of biological samples for proteomic analysis, and includes such processes as mixing, stirring, dispersing, or emulsifying in order to change sample's physical, but not chemical properties. Homogenization for proteomics incorporates five main categories: mechanical, ultrasonic, pressure, freeze-thaw, and osmotic/detergent lyses as detailed below.

Mechanical homogenization for tissues and cells can be accomplished by devices such as rotor–stator, and open blade mills (e.g., Waring blender and Polytron), or pressure cycling technology (PCT) such as French presses [6, 7]. Rotor–stator homogenizers can homogenize samples in volumes from 0.01 mL to ~20 L depending on the tip and motor used. For optimum results, the tissue should be cut into slices, the size of which is slightly smaller than the diameter of the applied stator, as larger samples may clog generator's inlet, making it impossible to achieve effective homogenization [8]. Depending on the chemical resistance of a cutting tool, it is possible to homogenize samples under acidic or basic conditions in order to prevent degradation by endogenous enzymes [9]. Heat transfer to processed mixture is low to moderate and the process usually requires external cooling. Sample loss is minimal compared to PCT, where by means of a pressure-generating instrument (Pressure Bioscience, West Bridgewater, MA) alternating cycles of high and low pressure are applied to induce cell lysis [8]. Pressure homogenizer is effective for homogenizing eukaryotic cells as well as microorganisms in suspension. The PCT method was assessed in *Escherichia coli* cell suspensions with improved protein solubilization and electrophilic resolution [10]. It was also employed for fast purification of proteins from cells in culture [11], although molecules such as mRNA were lost [12]. The PCT is ineffective toward tissues or organs without previous preparation

in any other type of homogenizer [8]. Liquid homogenization using Dounce homogenizer and Potter–Elvehjem homogenizer at low temperatures has been successfully used [13]. Blenders can be used to homogenize in either dry, or liquefied samples [8].

Mechanical homogenization may result in loss of activity of some proteins that are sensitive to heating and cooling during the process as was observed when dispersing human breast cancer tissue and calf uterus, resulting in rapid denaturation of the estrogen, and progesterone receptors; in such a case, use of detergent lyses at low temperature did not result in a significant loss of biological activity [14]. In case of unsatisfactory results, another type of homogenizers (*i.e.*, ultrasonic) could be employed to achieve an optimal homogeneity [15].

Ultrasonic homogenizers (also known as disintegrators or sonicators) are based on the piezoelectric effect that generates high-energy ultrasonic wave resolved after explosion/implosion of gas microbubbles, while interacting with the sample, to effectively destroy solid particles as well as cells. Although, this type of homogenization did not affect enzymatic activity of 13 investigated enzymes in leukocytes [16], the procedure may, however, lead to the disruption of noncovalently bound molecular clusters such as multienzyme complexes [17]. Ultrasonic devices are mostly effective to homogenize small pieces of soft tissues, as tough and dense tissues are not suitably homogenized by this method [8].

Freeze-thaw homogenization uses the destructive effect of ice crystal formation during the freezing process. The method is relatively fast, effective, and does not introduce external impurities into the sample because no frozen solution comes from an external environment. This method is effective toward most of the bacteria, plant and animal cells in water solution, and may be used as an additional or a final step after mechanical or ultrasound homogenization [8], although some microbial cells preconditioned in starvation media, such as *Vibrio parahaemolyticus*, are resistant to this method [18]. Moreover, the possibility of inducing changes in activity or properties of bioactive molecules (*e.g.*, enzymes and membranes) after few freeze-thaw cycles was noticed in case of G-protein coupled receptor kinases, β -arrestins [19].

Osmotic and detergent disruption of cells' walls and membranes of erythrocytes as well as homogenization of nuclear and mitochondrial membranes in cell extracts was reported. It was found useful for RNA extraction from the bacterium *Brucella abortus* internalized in macrophages [20], or for determining survival of *Staphylococcus aureus* after phagocytosis by human granulocytes [21]. Osmotic lyses were reported for microbial cell disruption, as in *Staphylococci*, after addition of lysostaphin to a hypertonic solution [22], or addition of lysozyme to the buffer in case of *Pseudomonas* species and other bacteria

[23]. The most commonly used detergents are Triton X-100, Tween 80, NP 40, and Saponin [8].

2.1.2 Protein solubilization

Proteins in biological samples are generally found in their native state associated with other proteins and often integrated as a part of large complexes, or into membranes. Once isolated, proteins in their native state are often insoluble. Breaking interactions involved in protein aggregation (*e.g.*, disulfide hydrogen bonds, van der Waals forces, ionic and hydrophobic interactions) enables disruption of proteins into a solution of individual polypeptides, thereby promoting their solubilization [24]. However, because of the great heterogeneity of proteins and sample-source related interfering contaminants in biological extracts, simultaneous solubilization of all proteins remains a challenge. Integration of proteins into membranes, and their association and complex formation with other proteins and/or nucleic acids hamper the process significantly. No single solubilization approach is suitable for every purpose, and each sample and condition requires a unique treatment. Sample solubilization can be improved by agitation or ultrasonification, but an increase in temperature must be avoided. The selection of the appropriate solubilization protocol and buffers has especially been facilitated by the availability of commercial kits [13], although it is somewhat more expensive than routine reagent methods.

Detergents within a 1–4% concentration range, to prevent hydrophobic interactions, are used to solubilize proteins. In sample preparation for 2-DE, only neutral (octylglucoside, dodecyl maltoside, Triton X-100), or zwitterionic (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS), CHAPSO, SB 3-10, SB 3-12, and ABS-14) detergents are used due to their compatibility with the separation mechanisms. The anionic detergent SDS improves solubilization but interferes with the first dimension separation, and must be removed if present in the preparation; commercial kits are available for this purpose (*e.g.*, ETTan™ 2D Clean Up kit, GE Health Care; ProteoSpin™ Detergent Clean UP micro and maxi kits, Norgen Biotek Corporation) [25].

To avoid protein modifications, aggregation, or precipitation resulting in occurrence of artifacts and subsequent protein loss, sample solubilization process necessitates the use in sample buffer of: (a) chaotropes (urea, thiourea, charged guanidine hydrochloride, GdnHCl) that disrupt hydrogen bonds and hydrophilic interactions enabling proteins to unfold with ionizable groups exposed to solution; (b) ionic, nonionic and zwitterionic detergents (SDS, CHAPS, or Triton X-100); (c) reducing agents that disrupt bonds between cysteine residues and thus promote unfolding of proteins (DTT/dithioerythritol (DTT/DTE) or tributylphosphine (TBP) or tris-carboxy-

ethyl phosphine (TCEP)); and (d) protease inhibitors [26]. In the presence of chaotropes, proteins are denatured and they easily aggregate and precipitate. The presence of urea and thiourea, and detergents helps maintain them in solution. In the past, urea at concentrations of 8–9 M was solely used, but it was found that the number of proteins solubilized increases when thiourea at a concentration of 2 M was added to 5–9 M urea. Heating of urea and thiourea containing solutions must, however, be avoided to prevent their hydrolysis and undesirable side reactions. GdnHCl is not compatible with the IEF process [13]. After disulfide reduction with reducing agents, TBP or TCEP, it was found that in order to prevent further oxidation, the newly produced free sulfhydryl group (SH) needs to be protected by alkylation, such as by addition of iodoacetamide when 2-DE separation is employed, because proteins' electrical charge – and hence their *pI*s – are maintained using this reagent [13].

Although there is no general procedure to select an appropriate detergent, nonionic and zwitterionic detergents such as CHAPS and Triton X series are less denaturing than ionic detergents, and have been used to solubilize proteins for functional studies. On the other hand, ionic detergents are strong solubilizing agents that lead to protein denaturation. However, sodium cholate and deoxycholate are soft detergents compatible with native protein extraction, although variables like buffer composition, pH, salt concentration, temperature, and compatibility of the chosen detergent with the analytical MS procedure, and how to remove it (by dialysis for example) all are crucial factors that need to be considered. Usually, tissue disruption and cell lyses require the combination of detergent and mechanical methodologies [13]. The proper use of above reagents, together with optimized cell disruption method, dissolution, and concentration techniques collectively determines the effectiveness of proteome solubilization methodologies (<http://www.mnhn.fr/mnhn/by/eDMS/2DE.pdf>).

2.1.3 Protection from proteolysis

It is estimated that nearly 7000 proteases and their homologs defined in the human genome may be related to one of the metalloserine-, cysteine-, or aspartyl-protease groups. If not inhibited, liberated/activated endogenous proteases, during cell membrane disruption for example, could lead to enzymatic protein degradation producing artifacts that complicates further analysis [8]. Protein degradation could be minimized by quick and small-scale tissue extraction, boiling sample in SDS buffer with high-pH Tris base, or by lowering the pH and performing ice-cold (20% TCA) precipitation [27]. Alternatively, denaturation in boiling water, focused microwave irradiation, or use of organic solvents may be used [28]. While active in high concentration of urea, proteases may be effectively inhibited by the presence of thiourea

in the lyses solution [29]. Heat shock proteins (sHsps) were found to protect proteins *in vitro* from proteolytic degradation [30]. It has been recommended to add specific protease inhibitors such as PMSF, aminoethyl benzylsulfonyl fluoride (AEBSF), ethylene diamine tetra acetic acid (EDTA), pepstatin, benzamidine, leupeptin, aprotinin, or cocktails thereof with a broader activity spectrum during cell disruption and subsequent preparation [8].

Although most protein electrophoretic separations are carried out under denaturing conditions, sometimes it may be necessary to preserve the native protein conformation in order to obtain detailed information on protein function and their possible interactions. In such a case, separation under native conditions in 2-DE is imperative [25]. When IEF during 2-DE was carried out for protein extraction, degradation was considerably increased, which could be prevented by using cup loading to apply protein to the strip [31], and carefully apply protease inhibitors in the reswelling buffer as they may modify proteins, or introduce charge trains and adducts [32]. As for LC separation, protease inhibitors as GdnHCl have been added both in binding and elution buffers maintained at 0–4°C [33]. Common protease inhibitors include PMFS, AEBSF, EDTA, or ethylene glycol-bis (2-aminoethylether)-*N,N,N',N'*-tetra acetic acid (EGTA), tosyl lysine chloromethyl ketone (TLCK), or tosyl phenyl chloromethyl ketone (TPCK) and benzamidine. Commercial preparations are also available such as Ettan Protease inhibitor mix (GE Health Care) and Halt Protease Inhibitor (Pierce) [13].

2.1.4 Removal of contaminants

Salts, buffers, detergents, nucleic acids, polysaccharides, lipids, and particulates frequently present in sample solutions often tend to interfere with protein separation steps, inhibit the digestion process, collide with MS analysis, or complicate statistical analysis; therefore, there is a need to remove these contaminants at a proper time during the analysis [34].

Salt migrates away from proteins during IES contributing to their precipitation and aggregation. Moreover, a high electric current carried by the salt load could interfere with electrophoretic separation of proteins and reduces the efficiency of 2-DE, and produces an elevation of temperature during IEF [13, 35]. Thus, if present in concentrations >100 mM, salts should be removed prior to IEF, although cup loading in 2-DE tolerates a slightly higher salt concentration. It is also possible to dilute sample below the critical concentration and apply larger sample volume on the IPG gel. Sample dilution is also recommended prior to CE provided that proteins of interest are available at detectable concentrations [36]. Salt removal is often accomplished *via* dialysis using either dialysis bags as in the traditional procedure, which is

time consuming, or employing spin microdialysis, ultrafiltration, gel filtration, precipitation with TCA or organic solvents, and SPE [8]. Other alternatives are the use of commercially available clean-up kits such as Ettan™ mini dialysis kits (GE Healthcare), Zeba Sephadex and microdesalt spin columns (Pierce), ProteoSpin™ CBED micro- and mini-kits (Norgen Biotech Corporation) [37].

The efficiency of four desalting procedures (desalting column packed with Sephadex G-100, on-target washing, centrifugal filter devices, and C₁₈ microcolumns) showed that for intact proteins, the best procedure was the application of C₁₈ microcolumns, pipette tips, and centrifugal filter devices [38]. A method for extraction of proteins from human body fluids (plasma, urine, amniotic fluid, and tears) employed the use of a centrifugal filter device and a sample buffer containing CHAPS for efficient lipid and salt removal [39]. Other techniques for salt removal included fast protein LC (FPLC), desalting columns, SPE, ultrafiltration or dialysis [32], or an automated HT nickel and glutathione disks [40] for protein purification.

The rupture of cellular structures after disruption of a cell provokes the liberation of hydrolytic enzymes, mainly proteases, which begin to exert their action with a slow kinetic. These enzymes are usually resistant to denaturation, although solubilization of protein in strong denaturing agents may prevent their action. Nevertheless, the use of protease inhibitors in the solubilization buffers has been essential in most preparations [13].

Most common detergent removal methods include dialysis, gel filtration chromatography, hydrophobic adsorption chromatography and protein precipitation. For detergents with high CMC and/or small aggregation number, dialysis is usually the preferred choice. For a wide spectrum of detergents present in the sample, gel filtration can be applied; however, it results in a considerable sample dilution [8]. Ion exchange chromatography effectively excludes nonionic and zwitterionic detergents, although it was also successfully applied for SDS removal [41]. SDS can also be effectively removed with nanoscale hydrophilic phase chromatography [42] or acetone precipitation, especially if carried out at –20°C. Ceramic hydroxyapatite (HAP) chromatography was developed for the complete removal of SDS bound to soluble or membrane proteins [43]. For zwittergent removal, equal efficacy of gel filtration chromatography and a detergent affinity bead chromatography column was reported, which was slightly better than dialysis. SPE was found efficient in CHAPS removal for dilute protein solutions than the standard dialysis or the gel filtration methods [44]. Commercially available detergent precipitation or gels effective for binding and removal milligram quantities of various detergents from protein solutions were employed (e.g., Extracti-Gel® D Detergent

Removing Gel, and the SDS-Out™ SDS Precipitation Reagent Kit, Pierce; Ettan Out 2D clean-up kit, GE Healthcare; Proteo Spin Out Detergent clean-up micro and mini kits, Norgen BioTek Corporation). Hydrophobic adsorption employing the use of insoluble resin (e.g., CALBIOSORB™, Calbiochem) has also been employed to remove excess detergents [13].

Lipids are widely present in biological fluids such as plasma complexed with proteins and this interaction reduces protein stability and might affect their pI and MW. In addition, these lipids can complex with detergents leading to reduction in protein enrichment and/or separation efficiency. In case of 2-DE separation, buffers – including CHAPS – were reported to remove lipids and salts efficiently [8]. Precipitation in acetone or combination of TCA/acetone also removed lipids efficiently, although lipids' elimination was more effective when they were associated with proteins in membranes [13]. Precipitation employing ACN supplemented with 1% trifluoroacetic acid (TFA) and 1% *n*-nonyl-β-Δ-glucopyranoside was helpful in dissolving membrane proteins and lipids [45].

Delipidation of human serum lipoprotein by use of RP C₁₈ SPE cartridges was reported to produce a higher, more reproducible, and a faster protein yield and desalting than commercial liquid–liquid methanol diethyl ether delipidation of lipoproteins for mass spectrometric analysis of the proteome [46].

The presence of polysaccharides, especially if they are charged, should be avoided because they can aggregate, clog the pores of polyacrylamide (PAC) gels in 2-DE leading to either precipitation or extended focusing time, complexing of proteins and/or horizontal streaking. Moreover, they bind positively charged proteins, and interfere with protein migration during electrophoresis [47]. The disturbances caused are similar to those produced by nucleic acids. Protein precipitation with TCA, acetone, ammonium sulfate, or phenol/ammonium acetate followed by ultracentrifugation was found to be effective in removal of polysaccharides. Commercial precipitation kits for removal of polysaccharides found in protein samples makes the procedure easier and faster [13].

Nucleic acids interfere with carrier ampholites and proteins, leading to poor recovery of DNA- or RNA-binding proteins; therefore, elimination of nucleic acids needs to be carried out by precipitation with TCA ultracentrifugation, or by digestion with protein-free DNase and RNase [26]. However, if DNases and RNases appear on 2-DE patterns, proteins tend to precipitate. Proteins associated with nucleic acids may be lost from the sample unless the nucleic acid fraction is extracted with a detergent cocktail [48]. Ultracentrifugation and addition of basic polyamine (e.g., spermine) was found effective in removal of large nucleic acids, as well as high MW pro-

teins. High ionic strength extraction and high pH extraction seemed to be potent in minimizing interactions between negatively charged nucleic acids and positively charged proteins [49]. A convenient alternative was reported that utilized QIASHredder (QIAGEN) and subsequent centrifugation [50].

To remove other contaminants such as small ionic molecules, nucleotide metabolites, phospholipids, either TCA/acetone precipitation or other salt-excluding techniques were effectively performed [51]. High-speed centrifugation is an alternative method [52]. Particulates must also be removed, as like nucleic acids they may plough the gel pores, which could be conveniently achieved by centrifugation [13].

2.1.5 Protein enrichment

Proteins concentration range in a sample such as blood is beyond the dynamic range of any single analytical sample. Therefore, prior to analysis, it is necessary to reduce sample's complexity by fractionation in order to enrich for proteins of interest (biomarkers in this review's context). During any enrichment process, conditions must be stable to avoid protein interactions among the rest of mixture components (*e.g.*, nonspecific interactions with other proteins). Prefractionation involves isolation of the sample into distinguishable fractions containing restricted numbers of molecules. This can be accomplished by many approaches including precipitation, centrifugation, LC, and electrophoresis-based methods, filtration, or equilibrium sedimentation. The selection of the technology strongly depends on the nature of the sample to be analyzed, the physicochemical properties of the proteins and their subcellular location, and the object of the study. It should be kept in mind, however, that there is no general enrichment protocol that exists for enriching low-abundant proteins (LAPs) [13, 25].

Selective precipitation employs acetone, TCA, ethanol, isopropanol, diethyl ether, chloroform/methanol, ammonium sulfate, PEG, and affinity precipitation [53, 54]. The precipitant ammonium sulfate causes protein destabilization, a phenomenon known as “salting out”. Addition of various organic solvents causes an increased attraction between particles of opposite charge in the sample solution leading to protein precipitation. Precipitate recovery relies on the sample redissolving in a smaller volume, followed by centrifugation or filtration. Immunoprecipitation is a more specialized approach that employs antibodies (Abs) selective for one or a group of proteins with similar epitopes (*e.g.*, phosphor or glycans) [55]. Separation of cell substructures can be accomplished by ultracentrifugation at different centrifugal forces in sucrose/mannitol gradient, which allows for separation of different cellular components (*e.g.*, membrane, mitochondrial, Golgi, nuclear, or other locally

abundant proteins) according to the density characteristics of the structure [56].

Electrophoretic enrichment methods employ gel separation in one- (1-DE) or two-dimensions (2-DE); the latter method enables simultaneous visualization of 100s of protein spots, their PTMs and quantification of protein levels, although separation of hydrophobic and membrane proteins, as well as alkaline and low molecular weight peptides poses severe limitations. Reproducibility of protein patterns between separate laboratories is difficult to standardize because of protocol variations, artifacts and technology limitations, as reviewed earlier [25].

IEF has emerged as a useful approach for protein prefractionation because of its high resolution, relatively short separation times, and its modest cost. Commercial isoelectric fractionation systems are available that perform focusing in solution-phase, off-gel and in-gel formats at the protein or peptide level. The nongel-based IEF methods have the advantage of convenience, as well as being able to accommodate large volumes, and thus are not limited by samples' amount [57].

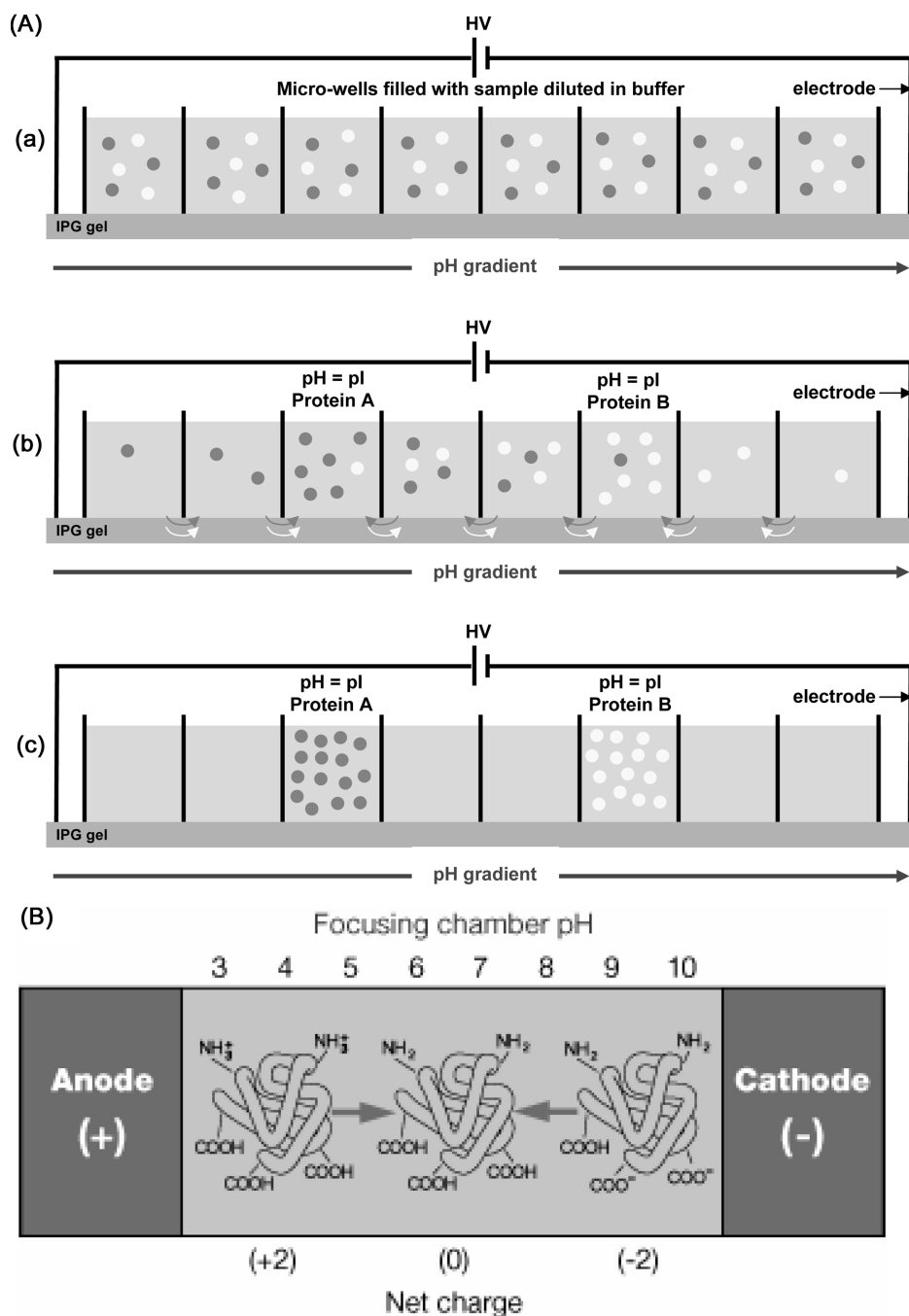
The OGEL fractionator from Agilent Technologies (Santa Clara, CA) uses a novel IEF method, which instead of performing focusing in free solution in the presence of carrier ampholytes, proteins and peptides are focused in an IPG strip, which is sealed against a multichamber frame containing sample and focusing solutions [58, 59] (Fig. 1A). During the separation, sample species migrate through the IPG gel and become focused according to their pIs. At the completion of focusing, proteins diffuse into the well adjacent to the section of the IPG strip within which they have been focused. This IEF approach reduces the risk of protein precipitation during focusing. For complex samples (plasma, serum, and cell or tissue lysates) total sample loads of 50 µg–5 mg are recommended. For simple mixtures, sample capacity is lower. The salt concentration of the sample should not exceed 10 mM. If startup voltage is low (below 100 V), this indicates that sample's salt concentration is too high. Typical focusing times are 12–24 h. At completion of focusing, the focused fractions could be recovered from each well *via* a pipette. This focusing permits harvesting a population of proteins having pI values precisely matching pH gradient of any IPG strip. Finally, due to the fact that only proteins cofocusing in the same IPG interval will be present, much higher sample loads can be accommodated, permitting detection of low-abundance proteins.

The first liquid-phase preparative IEF system (Rotofor) was introduced about 20 years ago by BioRad Laboratories (Hercules, CA) to fractionate proteins according to their pI, based upon the technology developed in Milan Bier Laboratory [60], and over the intervening two decades it has evolved into a family of systems ranging from the standard format to mini- and micro-formats. All three systems are multicompartiment electrolyzers that

share a common design in which the sample is separated into multiple fractions in a cylinder focusing chamber segmented into compartments by parallel monofilament polyester screens. The focusing chamber is connected to anolyte and catholyte chambers by cation and anion exchange membranes, respectively (Fig. 1B). These membranes isolate electrolytes from the sample in the focusing chamber, yet allow the current to flow for fractionation. The standard and mini Rotofor focusing chamber contains 20 compartments with 19 screens, while the microversion contains 10 compartments with 9 screens.

All three systems provide for cooling to dissipate heat during focusing. Run time for all three systems is typically 3 h. At the completion of fractionation, focused samples are harvested by penetration of the collection port seal by an array of needles, with vacuum-assisted transfer to collection tubes in the external harvesting station (standard and mini Rotofors), or to the internal collection tray (micro Rotofor).

A microscale liquid phase IEF fractionator (MicroSol-IEF) or the ZOOM IEF fractionator from Invitrogen Corporation (Carlsbad, CA) is based upon the method of Zou



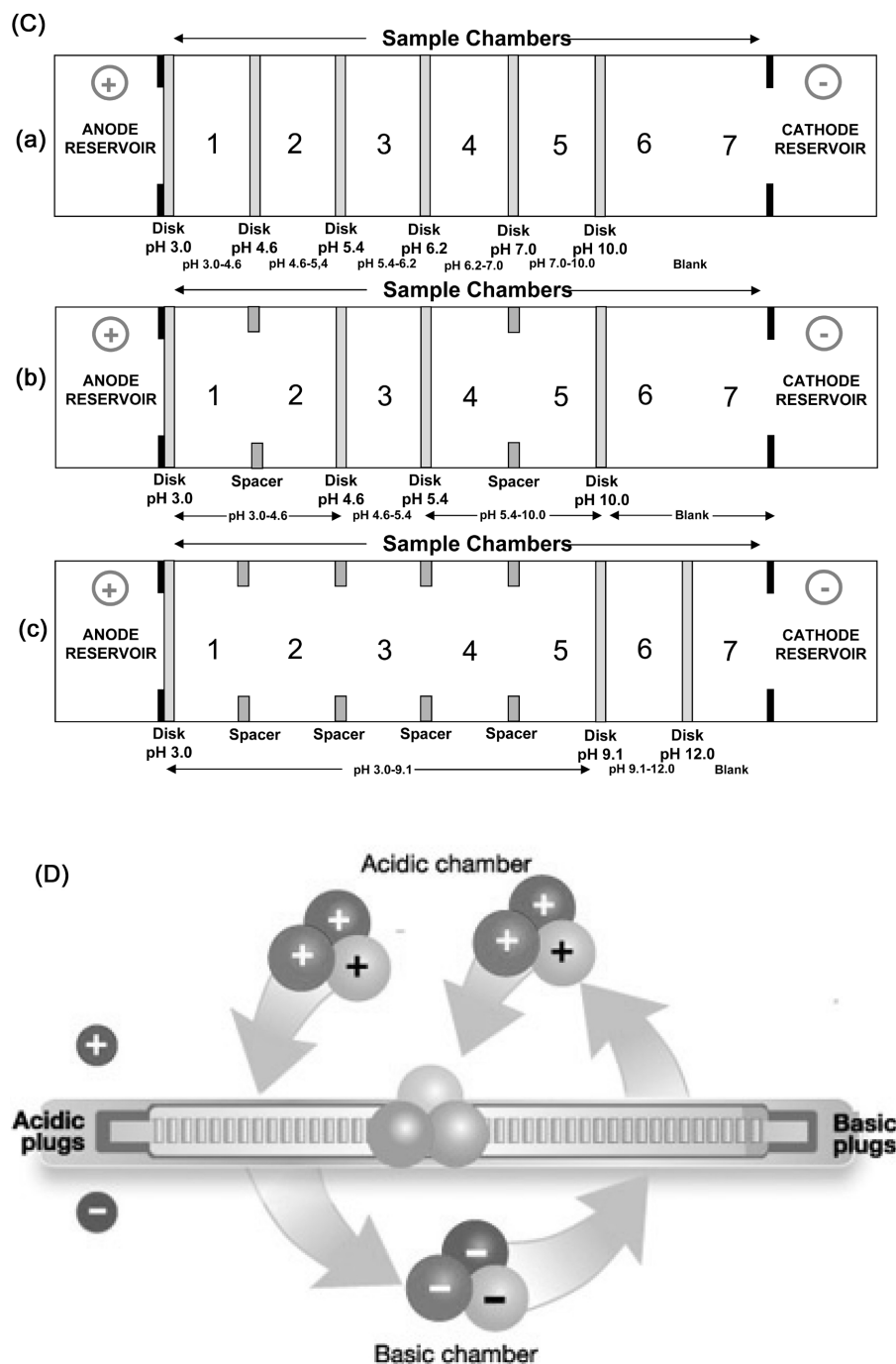


Figure 1. (A) Fractionation principle of the Agilent 3100 OFFGEL fractionator. (B) Separation principle for free-solution IEF in the BioRad RotoFor systems. A protein migrates in response to the electric field through the pH gradient established by the carrier ampholytes until it reaches the compartment where pH equals pI and becomes focused. (C) Formats for the Invitrogen Zoom IEF fractionator (a) standard format where six disks are used to create five fractions from pH 3.0 to pH 10.0, (b) extended format where seven disks are used to create six fractions from pH 3.0 to pH 12.0, and (c) three-disk extended format where three disks are used to create two fractions of pH 3.0–9.1 and pH 9.1–12.0. (D) Fractionation principle of the protein Forest digital Protein-Chip. Charged polypeptides migrate through the chip between the acidic and basic sides until they encounter a gel plug whose pH is at or near their pI. The uncharged polypeptide will no longer migrate and becomes trapped in these plugs. Courtesy of Tim Wehr, BioRad Laboratories (Hercules, CA).

and Spicher [61], in which a multicompartiment apparatus (4.7 mL total volume, 650 μ L/chamber) partitions complex protein samples on the basis of pH into multiple tandem electrode chambers divided by isoelectric membranes (ZOOM disks). Chambers can be set into specific pH ranges by buffers for fractionation of complex proteomes under denaturing conditions using solution-phase IEF in small sample volumes (Fig. 1C). Typical fractionation time is about 3 h, with a stepwise increase in voltage from 100–600 V. Because ~20% of the protein in each sample chamber remains adhered to the chamber walls, it has been recommended to add about 300 μ L of a denaturing solution to each chamber, place the chamber assembly on a rotary shaker for 10 min, and combine the wash solutions with the appropriate fraction, and fractions can then be harvested by a pipette [57].

The Digital Protein Chip (dPC) system recently introduced by Protein Forest (Lexington, MA) is a miniature electrophoretic device that employs parallel IEF for rapid fractionation and enrichment of complex protein mixtures [62]. The chip contains a linear array of 41 gel plugs, with 0.05 pH resolution between adjacent plugs (Fig. 1D). The chips allow placing ~2 μ L of acrylamido buffer into each chip feature, followed by UV polymerization. Three pH ranges are available: 4.20–6.20, 6.00–8.00, and 7.20–9.20. The dCP fractionator can accommodate up to six chips. Run time is 30–45 min, depending upon the fraction range. At the completion of focusing, gel plugs can be extracted from the chip for downstream analytical steps such as Western blotting or in-gel digestion followed by LC-MS.

Following fractionation of intact proteins by IEF, ampholytes and urea need to be removed prior to proteolytic digestion. This can be accomplished by TCA precipitation of the proteins, or by rapid desalting using a size-exclusion spin column. Focusing peptides is much easier than focusing proteins as peptides are natural ampholytes that will self-generate a pH gradient in the focusing chamber under voltage, which eliminates the problems of ampholytes in downstream analysis. Peptides are also soluble under focusing conditions, obviating the need for additives that would interfere with downstream LC-MS or MALDI-MS analysis. From bioinformatics standpoint, focusing at the peptide level complicates the picture because a given protein will exist as its component peptides and be defocused over multiple fractions, and therefore over multiple MS data files [57, 63].

A remedy to the slow migration of proteins in multicompartiment electrolyzers (MCEs) (Proteome Systems, Woburn, MA), in which a solution phase IEF is separated into discrete pH zones using membranes that can be set for specific pH ranges, due to the sieving effect of isoelectric membranes, was the introduction of hydrogel beads instead of membranes as pI barriers sandwiched in between the various chambers [64]. A composite isoelec-

tric beads made up of ionic acrylamide derivative monomers copolymerized with the pores of a central ceramic hard core thus minimizing mass transfer resistance of proteins that are transiently adsorbed onto the beads was employed. This method reduces separation time. Protein mixtures with species covering a large pI spectrum were fractionated by slicing out the extreme pI ranges (below and above predetermined pI defined by the pI of selected beads). The remaining protein mixtures (pI species between the previous pI of selected beads) were prefractionated under the same rules, thus yielding multiple fractions of predetermined pI ranges. Moreover, electrochromic chambers were separated by steric membranes with a cutoff of ~1000 Da, thus preventing the migration of species toward the electrochromic chambers [65].

Another multifunctional electrokinetic 2-D liquid-based, IEF prefractionation technology, the Gradiflow® System BF400 (French Forest, NSW, Australia) [66], comprises recirculating hydraulic flow of protein mixtures through a separation cartridge of two shallow separation compartments consisting of three polyacrylamide membranes located between two restriction membranes with an orthogonal electrophoretic transport of different proteins across a single separation uncharged membrane between the recirculating components. The Gradiflow technology operates on the principle of binary fractionations, by allowing for the electrophoretic separation of proteins based upon both a protein's charge and molecular shape (size) parameters, so that generally only two populations can be collected in each run: the upstream and downstream fractions [67]. Major drawbacks of the Gradiflow are the lack of sample loading flexibility (>8 mL volume), sharply defined molecular size cut-off, and potential protein loss at each membrane-based separation step according to pI and molecular weight [68].

The ProteomeLab® PF2D system (Beckman Coulter, Fullerton, CA) is an automated, 2-D fractionation system expressly designed for high resolution analysis of complex protein mixtures for down-stream proteomic analysis that uses IEF in the first dimension followed by non-porous RP-HPLC selectivity. This system has been shown to work effectively and reproducibly separating basic proteins that proved to be a challenge for a 2-DE [69], as well as highly hydrophobic microsomal proteins [70].

Another liquid-based IEF prefractionation technology developed about 50 years ago that works in the absence of either a stationary phase or a solid support gel material is a continuous free-flow electrophoresis (FFE) system for purifying cells and subcellular organelles [71]. FFE separates charged particles ranging in size from molecular to cellular dimensions according to their electrophoretic mobility or pI, where vertical free flowing buffer maintains a pH gradient across a single focusing chamber [72]. It continuously injects samples into a carrier ampholine solution flowing as a thin laminar film (0.3–

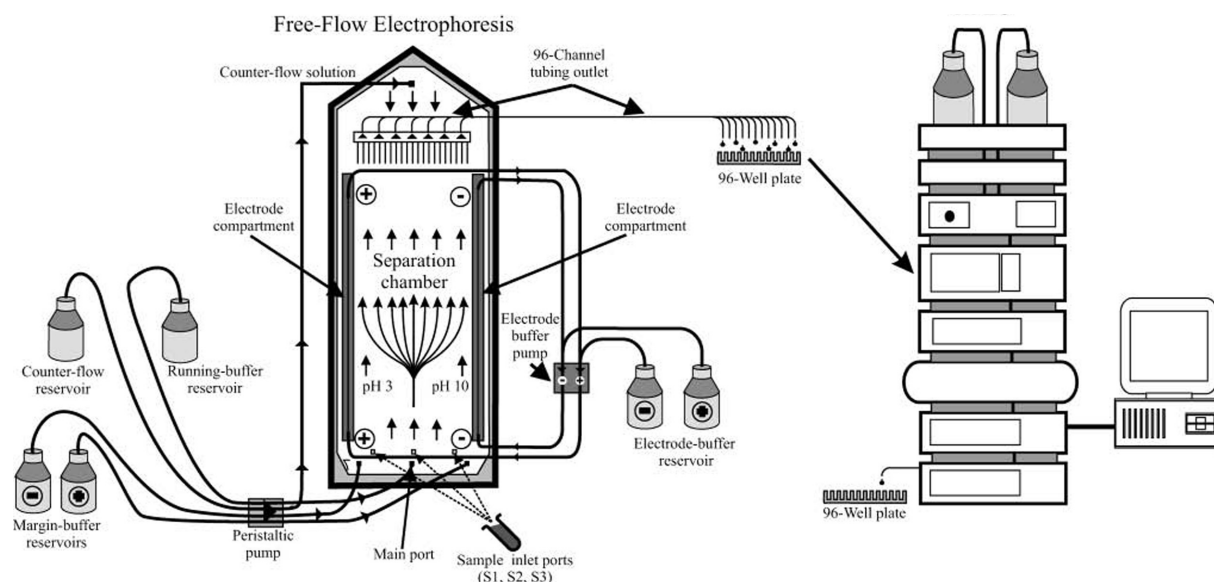


Figure 2. Schematic representation of the continuous FFE apparatus coupled offline to RP-HPLC. For analytical imaging separation, a portion of each first-dimension FFE-IEF fraction (50 μ L/total volume, (2 mL) was injected directly from the 96 deep-well plate using the Agilent 1100 HPLC equipped with a well-plate autosampler. From ref. [74]; with permission.

1.0 mm wide) between plates. By introducing an electric field perpendicular to the direction of flow, proteins can be separated by IEF according to their different *pI* values [73]. An off-line RP-1100 HPLC system (Agilent Technologies) can be coupled to a FFE device [74], as shown in Fig. 2. A 3-D image of a tryptic digest of a cytosolic extract of human colon cell line LIM1215 separated protein is shown in Fig. 3, whereas Fig. 4 shows a mucoprotein-depleted human urine that was subjected to both non-reducing 2-DE and the 2-D FFE-IEF/RP-HPLC system following a MS/MS detection method for comparison. Advantages of this system are: (a) protein or peptide separation in the first dimension (IEF), where very narrow range pH gradients are generated, are performed in a liquid phase, (b) the RP-HPLC stationary phase extends the resolving power of this 2-D system compared to other 2-D systems based solely on coupled HPLC columns, (c) the system is not sample limited, (d) separation efficiency is maintained by continual flushing of the separated sample, and (e) the system is capable of separating both peptides and proteins over a broad pH range. Early versions of FFE, such as RIEF and Rotofor, have evolved to the TECAN's FFE (now BD Biosciences, San Jose, CA). Regardless of the sample application method (continuous vs. interval), the applied electrical field causes charged sample components to move toward the respective counter electrode on the basis of electrophoretic mobility or *pI*. The advantages of FFE fractionation include good sample recovery, probably due to absence of gel media or membranous material, and higher sample loading capacity with continuous sample feeding. Additionally, reproducibility and high resolution is provided by throughput fractiona-

tion as samples can be collected in 96 fractions, which can enrich for proteins with specific pH range, and the capacity for native or denaturing electrophoresis [75]. Major drawbacks of FFE are that buffer constituents may interfere with MS measurements, leading to protein loss during buffer exchange [76]. Moreover, removal of buffer additives (e.g., glycerol) before each separation step is very cumbersome and leads to sample dilution [77].

Since 1944 several microfluidic, miniaturized FFE (μ FFE) devices (e.g., free-flow zone electrophoresis (FFZE), free-flow IEF (FFIEF), free-flow isotachiphoresis (FFITP), and free-flow field-step electrophoresis (FFFSE)) were developed to allow rapid separation (in seconds) and small volume (in μ L) with various modes. Eventually, such microfluidic FFE systems might find application in small portable devices and point of care tools [78]. An overall drawback of IEF prefractionation, in general, is that fractions collected contain high amount of ampholytes, which can, however, be effectively removed by microcolumns filled with C_{18} RP material [8].

The requirements for HT proteomic applications (e.g., hydrophobic interactions, immobilized metal affinity chromatography (IMAC), and affinity-based separations) have fueled the development of high speed separations [79], although they have been mostly used in the separation of peptides for shotgun proteomics, particularly continuous bed monolithic columns [80], which have the advantage of improved mass transfer properties, wide range of pore sizes, and low back pressure at high flow rates. Monolithic disks with ion exchange functionality were used to fractionate proteins from liver and hepatocellular carcinoma followed by analysis with 1-DE and 2-

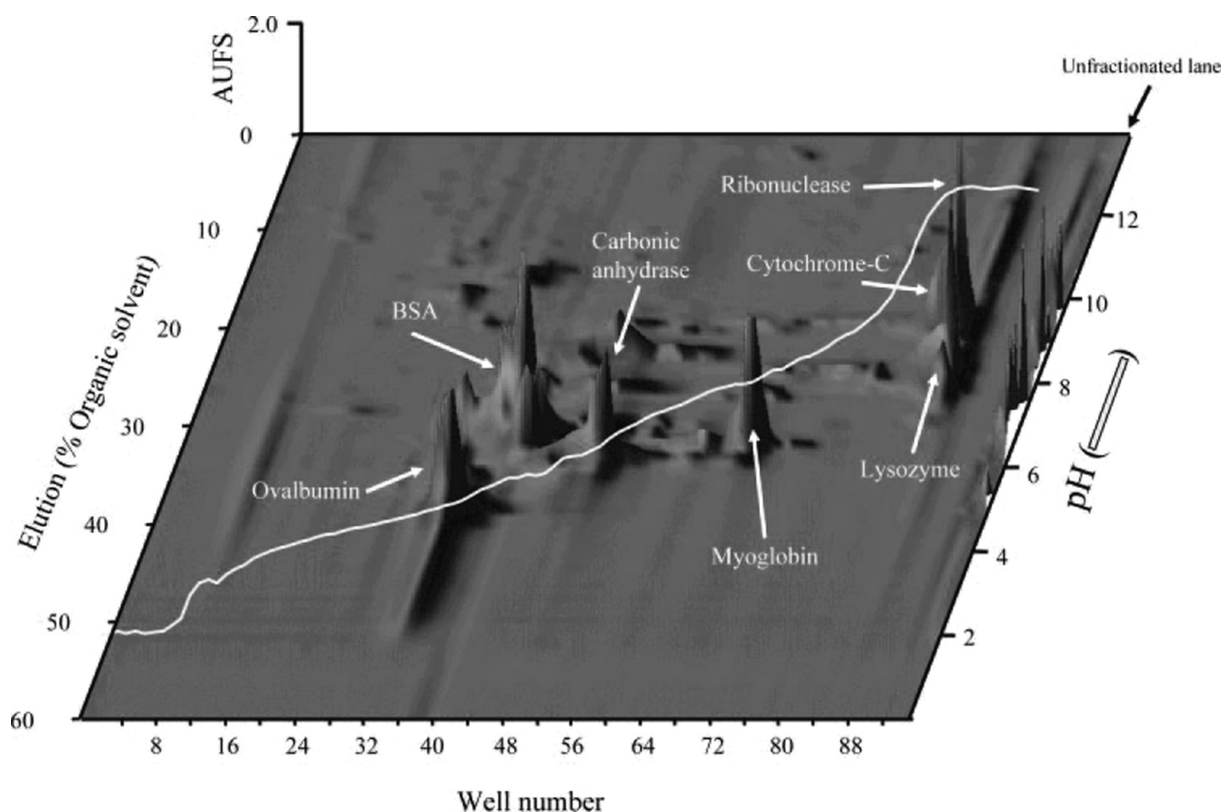


Figure 3. 3-D visualization of a native 2-D FFE-IEF (pH gradient 3–10)/RP-HPLC separation of standard proteins. Peak intensity (z-axis) absorbance plot at 215 nm. From ref. [74]; with permission.

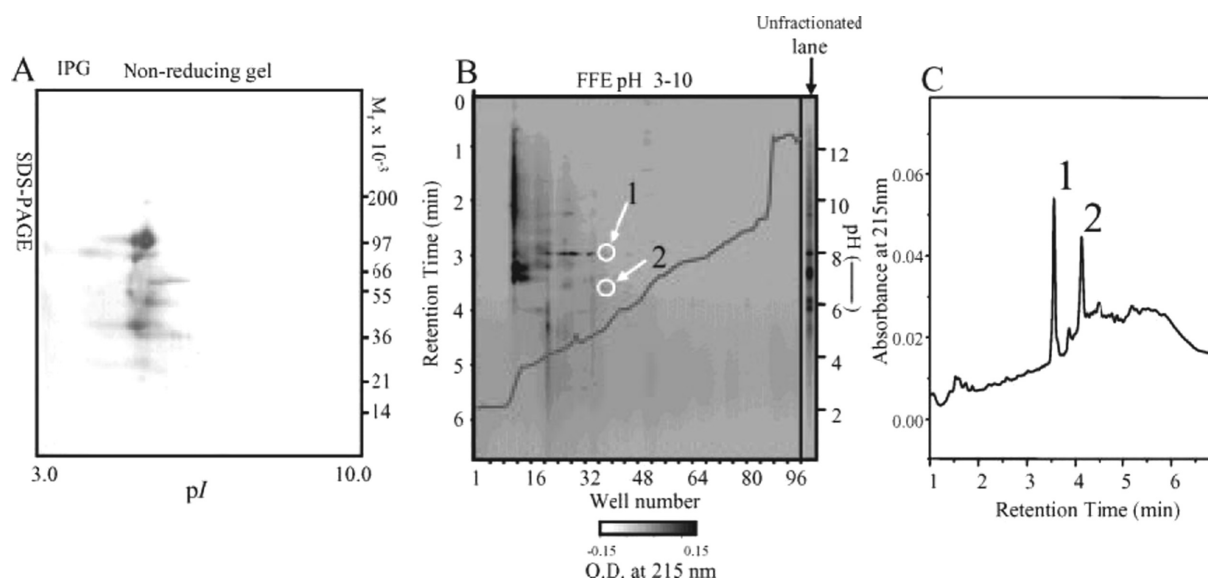


Figure 4. Comparison of human urinary protein separation by nonreducing 2-D FFE-IEF/RP-HPLC separation of 1.25 mg of human urinary proteins dissolved in 5 mL of IEF running buffer containing 0.2% w/v HPMC, 0.4% v/v Servalyte pH 3–10. (A) Nonreducing 2-DE. (B) FFE-IEF/RP-HPLC, pH 3–10. (C) RP-HPLC chromatogram of FFE fraction 42, pH 5.26, from circled peaks 1 and 2 in panel B, identified by N-terminal Edman degradation as CD59 gpi-anchored membrane protein, and spasmodic polypeptide, respectively. From ref. [74]; with permission.

Table 1. Instrumental techniques

Instrument	Principle	Manufacturer
Offgel OGE/IEF electrophoresis	A free-flow protein purification method based on isoelectric electrophoresis without the need of carrier ampholytes, in which protein solutions are flowed under an immobilized pH gradient gel (IPG) where an electric field is applied perpendicular to the direction of flow	Agilent Technologies
Rotofor	Proteins are separated in free solution by liquid phase IEF	BioRad Labs
Gradiflow BF 400	A size/charge IEF method using polyacrylamide membranes and tangential flow allowing binary fractionation to collect up- and down-stream populations	Frenchs Forest
Multichannel electrolyte IEF/Digital Protein Chip	Solution phase IEF separated into discrete pH zones using isoelectric membrane disks, or gel plugs, that can be set for certain pH ranges	MCE, Protein Forest
Microscale (Zoom) IEF	Electrode chamber with narrow range immobilized IPG gradients formed using precast polyacrylamide disks	Invitrogen Corporation
PF 2-D LC	An automated 2-D liquid-phase fractionation system using chromatofocusing and nonporous RP-HPLC selectivities	BioRad, Beckman-Coulter, Lumicyte
Free-flow electrophoresis (FFE)	Sample injected into a separation chamber of two parallel plates, then transported within a 0.5 mm of aqueous film formed between the two plates that are flanked by an electric field perpendicular to the laminar flow. Charged particles are deflected allowing for separation and collection of 96 fractions	BD Biosciences
Biosensor surfaces	Biosensor surfaces functionalized with specific proteins are used to affinity purify binding partners and their complexes, which can then be isolated for downstream analysis	BIAcore, IAsys, Vir, Genoptics

Modified from refs. [57, 66].

DE, surface-enhanced laser desorption ionization (SELDI) and electrospray MS/MS [81].

The development of lab-on-chip technologies, in which channels are etched onto glass or polymer chips [82, 83], or protein arrays that are coated or immobilized in a grid-like pattern on small surfaces [84], as well as nonporous magnetic particles have greatly reduced nonspecific adsorption compared with conventional chromatographic supports [85]. The various instrumental techniques detailed above are presented in Table 1.

2.2 Approaches relying on biological characteristics

Approaches relying on biological characteristics include prefractionation into cellular compartments (e.g., cytoplasm and membranes), subcellular compartments and organelles. They are simpler and more focused than physicochemical ones discussed in the previous sections, and can considerably increase our understanding of the proteome.

2.2.1 Subcellular fractionation (SF)

SF is the first step among enrichment techniques for reduction of sample complexity, which is of importance for analysis of intracellular organelles (e.g., nucleus, mitochondria, Golgi apparatus, lysosomes, exosomes, peroxisomes, and phagosomes) and multiprotein complexes. SF is most efficiently combined with 2-DE-MS analysis as well as with gel-independent techniques. SF allowing fractionation of organelles consists of two main steps: (a) disruption of the cellular organization (homogenization) and (b) fractionation of the homogenate to separate the different populations of organelles. Centrifugation is a traditional method for organelle isolation [86], and is compatible with further steps in protein solubilization and separation. Problems with reproducibility are frequent when protein depletion technologies are used [87]. Cells are collected by a low-speed centrifugation step and mechanically homogenized. After homogenization, the nuclei are removed by a low-speed centrifugation and can be purified from the pellet that contains cell debris and unbroken cells for further analysis. The postnuclear supernatant (PNS) contains the cytosol and

other organelles in free suspension, which can be subsequently separated by differential gradient centrifugation [88]. Although time consuming, labor intensive and resulting in dilute fractions, centrifugation is commonly used. While differences in composition of subcellular components affect relative densities of fractions, the degree of separation also depends on the gradient medium used. Sucrose is the most used medium [64], although other reagents such as Ficoll, Percoll, Nycodenz, or Metrizamide have been employed. Other techniques like FFE or immunoisolation have also been applied to SF of organelles [89]. Sometimes after centrifugation, an additional precipitation step may be required, especially when dealing with plant tissue [13].

Ideally, the purity of isolated organelles is important for the comprehensive analysis of total organelle proteome, although complete purification is often impossible for functional proteomic studies because cross contamination of fractions is a significant problem, which can be monitored by the use of appropriate markers. Therefore, enrichment of some organelles or certain subcellular fractions could be beneficial for the detection of low abundant proteins (LAPs) and tracking of their changes after stimulation of cells [90]. As an example, the stacked Golgi fraction from rat liver was fractionated using two sucrose step gradient centrifugation, followed by trypsin digestion, and the peptides were subsequently separated by multidimensional protein identification (MudPIT) technique. Forty-one proteins were discovered, two with confirmed Golgi location, and an arginine dimethylation species was identified on 18 of these proteins alluding to the role of methylation in Golgi function [91]. Peroxisomes were isolated from rat liver by homogenization followed by using the Nycodenz density gradient centrifugation, and organelles purified by immunosonication with Abs to peroxisomal membrane protein (anti-PM70) bound to magnetic beads. Purified peroxisomal fraction 34 was separated by 1-DE to visualize proteins that were identified after trypsin digestion by LC-MS [92].

Bioinformatics analysis of 3962 proteins of the yeast *Saccharomyces cerevisiae* previously localized by green fluorescent proteins (GFP) tagging and microscopy to 22 subcellular organelles or compartments pointed out that different compartments showed significantly different distributions of protein pI and hydrophobicity, with mitochondrial and endoplasmic reticulum (ER) proteins showing the most differences to other organelles to these two experimental parameters [93]. Therefore, for proteins to be separated and identified efficiently, analytical strategies need to be employed that pay careful attention to the degree of acidic, basic, hydrophobic, and hydrophilic proteins in each compartment [94].

Because of limited resolution of the available fractionation methods, it is often difficult to isolate and thus

profile pure organelles for identification of the protein components of specific fractions containing the target organelles using MS. Moreover, many secretory proteins are often observed to dramatically shuttle between organelles. Therefore, determination of their true cellular localization requires the concurrent analysis of multiple organelles from the same cell lysate. An integrated experimental approach that simultaneously profiles multiple organelles (e.g., ribosome, mitochondria, proteasome, lysosome, ER, and Golgi apparatus) based on the SF of cell lysates by density gradient centrifugation, isobaric tags for relative and absolute quantification (iTRAQ™) labeling and MS analysis of proteins in selected fractions, followed by principle component analysis (PCA) statistics of the resulting quantitative proteomic data, has been carried out using that approach [95].

2.2.2 Purification of protein complexes and microdomains

Proteins rarely function in isolation, but are rather organized in functional units that are different in size, number of interacting partners and stability, ranging from huge stable ribosomes or nuclear pore domains to small and transient signal transduction complexes. Studying of these multiprotein complexes and microdomains provides information about the spatio-temporal organization of signal transduction or metabolic processes within a cell as a major part of this information is lost when cells are lysed and proteins digested before analysis. Because isolated protein complexes have much reduced complexity, this allows for identification of low copy number proteins present in the complex and connecting them to particular function [90]. Multiprotein complexes and associated proteins can be isolated and purified by a variety of techniques such as affinity-based, recombinant pull-downs, LC, blue native gel electrophoresis (PN-PAGE), 2-DE/LC/CE and FFE methods, followed by MS analysis [86, 89, 96–98].

In a rat study aiming at targets for tissue-/organ-specific delivery of therapeutic and imaging agents *in vivo*, tissue subfractionation with subtractive proteomics and bioinformatics analyses reduced tissue complexity by more than five orders of magnitude and unmasked a subset of proteins at the blood tissue interface [90].

2.3 Sequential extraction method

An optimized sequential extraction method for fractionation of proteins in their native state according to their subcellular localization yielded four subproteomes enriched in: (a) cytosolic proteins, (b) membrane and organelle-associated proteins, (c) soluble and DNA-associated nuclear proteins, and (d) cytoskeletal proteins, respectively. Four extraction buffers of defined ionic and osmotic composition containing surfactants enabled

stepwise disintegration of cells and selective extraction of certain subcellular components. This method allowed for the assessment of spatial rearrangements of signaling proteins, as demonstrated on signal-dependent redistribution of phosphorylated mitogen-activated protein kinase (MAK) and nuclear factor kappa B (NF κ B), between cytoplasm and nucleus [99].

2.4 Membrane proteins' extraction

Membrane proteins are important for proteomics as they represent a large population of the proteome in the form of receptors, transporters, channels, and a variety of cellular mechanism, which makes them a major target of pharmacological interest. However, membrane proteins are still under identified and underrepresented during whole cell proteome analysis [8]. Although membrane proteins with up to 12 transmembrane α -helices have been resolved and identified by 2-DE-MS [100], most membrane proteins have been resistant to this approach.

Membrane proteins are often enriched by ultracentrifugation in sucrose gradient, lectin affinity chromatography in combination with centrifugation, silica beads or biotinylation and interaction with immobilized streptavidin [101]. Solubilization of this fraction is accomplished by using detergents, whose choice depends on the nature of the experiment. Combinations of chloroform and methanol were used to extract hydrophobic chloroplast membrane proteins [102]. The aqueous two-phase system, at least one of them containing a water-soluble polymer, which employed detergent DDM, Triton X-114 or PEG for the selective binding of one or more proteins of interest to the one of the incompatible aqueous phases, was used for membrane enrichment [103, 104]. A combination of chloroform and methanol was employed for differential extraction of membrane proteins from chloroplasts [105]. Centrifugal sucrose gradient fractionation was employed for isolation of mitochondrial membranes [106]. Identification of membrane proteins has been further complicated by the lack of tryptic cleavage sites across transmembrane chain fragments. Enzymatic digestion often results in large, hydrophobic species, which hinder identification. To enlarge sequence coverage, a mixture of proteases and cyanogens bromide with addition of detergents was carried out [107, 108].

Analyses of proteins from a number of proteomic studies of cell membranes have demonstrated that a significant component of the identified proteins is not predicted to contain transmembrane regions. The presence of such proteins may arise as a result of contamination of the membrane preparations or through real associations. Identification of integral proteins, as well as those that are intimately associated with the microsomal membranes of K562 cells, first necessitated removing noncovalently associated peripheral proteins and the

residual proteins were then 1-DE separated and analyzed by LC-MS/MS. Tandem lectin affinity was also examined as an approach for the enrichment of membrane glycoproteins. Approximately, 41% of the isolated proteins were assigned as membrane proteins based on the presence of transmembrane regions or covalent PTMs that could account for membrane association. Collectively, these results indicate that there is a significant component of nonintegral proteins that appear to be as closely associated with membranes as integral elements [109].

Plasma membranes (PMs) are important for cells as they form a selectively permeable barrier to the environment, and many essential tasks of PMs are carried out by their proteinaceous components, including molecular transport, cell–cell interactions and signal transduction. Because of their low abundance and immense heterogeneity, they require special treatment in order to identify and characterize them. An effective tool for PM isolation is partitioning in aqueous polymer two-phase systems (e.g., PEG and dextran) in which membranes are separated according to differences in surface properties, rather than size and density. The main advantages of this method are the high yield and purity, together with rapid processing. Different factors such as molecular weight and concentrations of the polymers and salts can be explored to optimize the partition behavior, so that an effective partitioning can be carried out within a few hours without employing specialized equipment [110].

Examination of membrane proteins of enriched and selectively isolated from microdissected ovarian tumor cells within the pellets was carried out by treatment with two different protein extraction methods that employed either SDS detergent or ACN organic solvent. The detergent-based preparation extracted proteins effectively from pellets, and was compatible with subsequent proteome analysis using capillary IEF/nano RP LC separation, coupled with nano ESI MS. Among proteins identified from an amount of pellet equivalent to 20 000 cells, 773 proteins were predicted to contain one or more transmembrane domains, corresponding to 22% membrane proteome coverage within the Swiss-Port Human protein sequence entries [111].

Aqueous polymer two-phase systems have been described for the preparative isolation of PMs [112]. Separation was attained due to differential affinity between two immiscible aqueous polymer phases. Qproteome Cell Compartment Kit[®] from Qiagen was used for separation of cytosolic, membrane and nuclear proteins, and Nuclear Subfractionation Kit for isolation of nucleic acid binding proteins from isolated nuclei [113, 114]. An immunoaffinity purification of PM with Ab superparamagnetic beads, following sucrose gradient separation of mouse liver PM, was described [115].

Two common methods have been used to isolate cholesterol-rich membrane microdomains with distinct

lipid and protein composition termed “lipid rafts” by treatment with either high pH or nonionic detergents followed by treatment with either high pH or nonionic detergents, and subsequent density gradient centrifugation; however, both methods have been plagued by contamination from nonraft proteins, which was overcome by using quantitative methods as stable isotope-labeling with amino acids in cell culture (SILAC) to determine the subset of cholesterol-dependent proteins depleted from rafts by cholesterol-disrupting drugs using a high-resolution MS method [116]. Seven-hundred and three detergent-resistant fractions proteins (of which 392 raft proteins) and 585 carbonate-resistant fractions were identified; among the rafts and raft-associated proteins a significant number of serine/threonine kinases/phosphatases, as well as numerous heterotrimeric G protein subunits, suggesting that rafts may be general signaling coordinators. Comparison of this data with previous published one on lipid rafts showed that only less than half of the 19 proteins in a detergent-resistant fraction from Jurkat T cells identified in a study [117], and approximately two-thirds of 70 proteins identified in another study [118] to be authentic raft proteins; the remaining being false positive. However, carbonate-resistant fractions were less specific for raft isolation and more difficult to interpret than detergent-resistant method [119].

Lipid rafts were originally defined as detergent-resistant membranes (DRMs) due to their relative insolubility in cold nonionic detergents. Recent findings suggest that although DRMs are not equivalent to lipid rafts, the presence of a given protein within DRMs strongly suggests its potential for raft association *in vivo*. A differential detergent extraction method employing 2% Triton X-100 was shown to enable rapid DRM isolation, minimized nuclear contamination and yielded fractions compatible with MS analysis [120].

Caveolin-enriched membranes were isolated by either cationic silica affinity purification or buoyant density methods. Subsequent 2-D separation followed by MALDI-TOF analysis showed improved identification of membrane proteins and their PTMs. On the other hand, cationic silica purification yielded predominantly ER, whereas the cold-detergent method yielded a large number of caveolae residents, including caveolin-1 [121].

An affinity-based isolation method was used to enrich and purify parts of blood vessel endothelial cells that contact the blood in organs such as lung and lung tumors by infusing colloidal silica particles into the blood stream of rats, where these particles attached to the endothelial cells, followed by centrifugation of tissue homogenates to separate endothelial cell membrane and the attached caveolae from the remainder of the cells. For purification, an Ab that recognized caveolin coupled to magnetic beads was used to isolate caveolae and their associated proteins. Purified caveolae displayed a greater

than 20-fold enrichment for specific markers. Analysis by 2-DE produced high-resolution vascular endothelial protein maps of major rat organs and showed 37 proteins to be present only in the endothelial membrane; 11 of which possess an extracellular portion that could be presented to blood cells. Expression profiling and γ -scintigraphic imaging with Abs suggested two of these cell surface proteins (aminopeptidase P and annexin A1) as selective *in vivo* targets for Abs in lungs and solid tumors, respectively. Radio immunotherapy targeted against Annexin A1 selectively decreased tumor size and increased animal survival [122].

A sequential fractionation strategy following homogenization of cells by centrifugation of the postnuclear supernatant at $100\,000 \times g$ that separated total membrane fraction from cytosol allowed extraction of peripheral membrane proteins from membrane pellet in 0.1 M sodium carbonate, pH 11.0; the remaining integral membrane proteins were analyzed [123]. Alternatively, Triton X-114 was applied to enrich for the integral membrane protein fraction [124].

A general MS-based proteomic “shave-and-conquer” sequential extraction strategy was used that targets specifically glycosyl phosphatidyl inositol-anchored proteins (GPI-APS) from *Homo sapiens* and from *Arabidopsis thaliana* that act as enzymes and receptors in cell adhesion, differentiation and host–pathogen interactions and are potential diagnostic and therapeutic targets. Raft-enriched membranes of human HeLa cells were purified by homogenization of cells and ultracentrifugation in sucrose gradients. After extraction of peripheral membrane proteins by sodium carbonate, lipid rafts were obtained from membrane fractions by two-phase separation in the presence of Triton X-114. The isolated membrane fractions were treated with phosphatidylinositol phospholipase C, which hydrolyzes phosphatidylinositol, releasing the soluble GPI protein from membrane/detergent phase and allowing its recovery in the aqueous phase. Proteins were then separated by 1-DE and identified by MS. Six GPI-APs were identified in *H. sapiens* lipid raft-enriched fraction and 44 GPI-APS in an *A. thaliana* membrane preparation [125].

3 Sample preparations from biological fluids

3.1 Body fluids

Many of the diagnostic, prognostic, or monitoring response to therapy biomarkers used in clinical practice are found in biological fluids (the most widely used ones are blood and urine, which are easier to access compared to tissue biopsies, besides being minimally invasive). Body fluids are very complex mixtures of molecules with a wide range of polarity, hydrophobicity, and size over a

range of several orders of magnitude. Ideally, a crude, unprocessed sample should be analyzed, which would avoid all artificial losses or biases arising from sample preparation. However, since all body fluids contain a large amount of different ions, lipids, carbohydrates, *etc.*, these samples generally cannot be analyzed in the native form in a mass spectrometer, and a pre-MS separation is a necessary prerequisite in order to cope with the complexity and dynamic range of these samples [25, 63].

3.1.1 Urine

Among body fluids, urine is especially attractive for biomarker discovery in urological diseases because: (a) it contains fewer proteins than, for example, blood because only a few organs are located directly along the path of urine production and excretion (*i.e.*, kidney, urinary tract, including bladder), (b) it can be obtained in large quantities using noninvasive procedures, (c) repeated sampling from the same individual is achievable, (d) it contains proteins and polypeptides of lower molecular mass (<30 kDa) that are highly soluble, which facilitates analysis of such polypeptides in their natural state without the need for additional manipulation (*e.g.*, tryptic digest) [126], and (e) for proteins >30 kDa, urinary polypeptides are stable and do not generally undergo significant proteolysis within several hours of collection, in contrast to blood where activation of proteases and the generation of an array of proteolytic breakdown products is often associated with its collection [127].

The urinary proteome appeared to be stable when urine was stored up to 3 days at 4°C, or up to 6 h at room temperature, probably because following its storage for several hours in the bladder, proteolytic degradation by endogenous proteases is complete by the time of voiding [126]. Urine, however, has disadvantages as a source for protein markers due to: (a) the wide variation in protein concentration, which is largely due to differences in person's fluid intake. This problem can be mitigated by standardization based on creatinine [128] or peptides present in urine [129], (b) inconsistency of its pH that may alter the activity of proteases in a fraction of the urinary proteome leading to greater variability of the proteome during the day due to factors such as different diets, metabolic or catabolic processes, circadian rhythm, exercise, and circulatory levels of various hormones [130]. However, the basal or housekeeping proteins of urine remains largely unaffected by these changes [129], and (c) clear differences between early stream and mid stream urine samples have been found [131]. Therefore, standardization of urine collection protocols for biomarker discovery is a must [132].

3.1.2 Blood

Plasma is the largest single component of blood, comprising about 55% of total blood volume. Plasma is

obtained from whole blood after addition of an anticoagulant (*e.g.*, citrate, heparin or EDTA) to prevent blood to clot, preferably in the presence of inert catalyst such as glass beads or powder. Although serum is a more convenient specimen, its protein profile is different from plasma [133], as an array of proteases are activated immediately upon clotting, resulting in the generation of many degradation products. Consequently, the Human Proteome Consortium has recommended that blood be examined as plasma rather than as serum and established standardized sample collection protocols [127].

Serum and plasma potentially contain elements of all proteins produced in the body, and studies suggest that the low molecular weight (LMW) protein/peptides in these fluids (*e.g.*, peptide hormones or small secreted proteins) are correlated with pathological conditions and present opportunities for potential clinical utility for diagnostic, prognostic, or predictive response to therapy markers [134]. The technical challenges in the analysis of plasma/serum proteome is that their proteins are present at unequal concentrations as a few are so dominant (*e.g.*, albumin and globulin present at almost 90% of total proteins by weight) that they mask the detection of other proteins, especially low abundant ones, which are considered to be of clinical importance [25]. The 30 most abundant proteins in the plasma of healthy persons, as well as the half-lives of some are presented in Table 2. Ranking these proteins by molar than by mass abundance changes the perspective on the relative abundance of many plasma proteins, particularly the small ones. Most larger proteins have half-lives of several days, while those with M_r of <30 000 Da are cleared by filtration in the kidneys, giving half-lives of a few hours, like retinol-binding protein, and are extended several folds by binding to a carrier protein such as albumin [135]. Without fractionation, the complexity of plasma/serum is overwhelming that important biological information will be in background noise and will not be detected by current available MS technologies [136].

Standardizing sample preparation procedures for plasma/serum profiling, including the type of collection tubes and coagulants, the clotting and incubation time before sample isolation, storage conditions, strategies used for removal of high abundant proteins (HAPs), as well as fractionation techniques employed either to generate several fractions or to selectively obtain a particular subset of peptides/protein, although time-consuming and error prone is critical for obtaining reliable biomarkers and building a biomarker pattern, since slight changes in a given sample preparation could lead to very different protein profiles [137].

3.1.3 Sample collection, handling, and storage

In spite of the impact of this parameter on the sensitivity, selectivity and reproducibility of the analysis, only a few

Table 2. Ranking of plasma proteins in healthy persons in order of molar or mass abundance, with examples of half lives^{a)}

No.	Protein rank	Concentration		Molecular mass (M_r) in Da	Half life
		M · mol/L	mg/L		
1	Albumin	500–800	35 000–52 000	66 438	15–10 days
2	IgG	40–100	7 000–16 000	160 000	
3	Apolipoprotein A-I	36–72	1 000–2000	28 079	
4	Transferrin	25–45	2 000–3600	79 600	7 days
5	Apolipoprotein A-II	22–60	200–550	8 691	5 days
6	α_1 -Proteinase inhibitor	18–40	900–2000	55 000	1–2 days
7	α_1 -Acid glycoprotein	12–30	500–1200	40 000	
8	Transthyretin	15–30	200–400	54 000	
9	Hepatoglobulin	3–20	300–2000	104 000	
10	Hemopexin	15	900	57 000	
11	IgA	4–24	700–4000	170 000	2.5 days 4 days
12	Apolipoprotein C-III	6–20	60–180	8 765	
13	α_2 -Macroglobulin	7–17	1 300–3000	30 000	
14	α_2 -HS glycoprotein	12	600	50 000	
15	Gc globulin	8–14	400–700	51 000	
16	Apolipoprotein C-I	6–12	40–80	6 631	
17	Fibrinogen	6–12	2 000–4000	340 000	
18	α_1 -Antichymotrypsin	7	500	68 000	
19	C3	5–10	900–1800	180 000	
20	b ₂ -Glycoprotein I	4–8	150–300	40 000	
21	Vitronectin	4–8	250–450	55 000	10–12 h
22	α_1 -B Glycoprotein	3–6	150–300	63 000	
23	Apolipoprotein A-IV	3–6	130–250	43 375	
24	Apolipoprotein C-II	2–7	20–60	8 915	
25	β_2 -Glycoprotein II	2–5	12–30	63 000	
26	Antithrombin III	3	200	65 000	
27	Inter α -trypsin inhibitor	2–4	400–700	160 000	
28	Plasminogen	2–4	150–350	81 000	
29	Ceruloplasmin	1.5–5	200–600	135 000	
30	Retinol-binding protein	1.5–3	30–60	21 000	

^{a)} Modified from ref. [135].

studies have been carried out that showed sampling procedures to have the greatest effect on proteome profiling, whereas handling procedures and storage conditions to have relatively minor effects [138]. However, standardized protocols for plasma/serum handling and storage are needed in order to have comparable results between different laboratories [139].

Studies on the effects of blood collection on many types of laboratory analyses [140] have shown that optimization and standardization of collection tubes is important for reliable analysis of plasma/serum proteins. Commercially available blood collection tubes contain multiple components (e.g., silicones commonly used for lubricants for stoppers or coatings for the internal surface of collection tubes; polyvinyl pyrrolidones or PEGs added as surface wettings; clot inhibitors or activators; substances in polymeric gels to separate blood constituents; polymers in rubber stoppers and plastic tubes) [141] that may interfere with MS analysis when they are shed into these body fluids. The kind of tube used can also influence adsorption of plasma/serum proteins to tubes' inner surfaces. Significant differences have been found

when using red-top tubes (glass tubes containing no preservatives or anticoagulants) *versus* tiger-top tubes (also known as serum separator tubes, SST) [138, 142].

The coagulant added to blood for making plasma influences MS protein profiles [143]. For example, although platelets are more stable in citrate anticoagulants, collecting tubes often contain a liquid form that dilutes the plasma. Heparin that binds to and enhances the activity of antithrombin III also binds to a number of other proteins [144]. Because the activity of many proteases requires metals, the chelation of EDTA prevents coagulation [126]. Freshly collected EDTA-treated blood is only slightly stable, but over longer periods of time, marked changes appear as elapsed time before centrifugation increases [126]. Plasma protein profiles obtained by EDTA treatment were most divergent from those obtained by citrate or heparin treatment [138], probably because EDTA leads to platelet clumping and aggregation that could change the protein content of plasma [145].

Clotting or time of incubation before separating blood cells from serum influences protein profiles. Profiles from plasma samples changed as time lag increased

Table 3. Strategies used for HAPs depletion and enrichment

Method	Principle	Advantages	Disadvantages
(A) Depletion			
Centrifugal ultrafiltration	Membrane filtration combined with solvents	Fast, easy to use, inexpensive	Potential loss of components binding to HMW proteins
SPE disk formats	Bases on ion exchange, metal chelating, affinity or dye ligands, bacterial protein A and G, or combination thereof	High selectivity, reproducibility and sensitivity when using series of different columns	High cost of Abs depletion columns, generally short lifetime (L), sample dilution or loss may occur, not all LAPs can be accessed by these columns
Disk plates SPE formats	Same as SPE columns	Highly suitable for HT and automation	Same as SPE columns
Organic solvents, extraction	Solvent precipitation of HAPs with simultaneous extraction of LAPs	Ease of use, convenience	Requires organic solvents, leads often to sample dilution
(B) Enrichment			
Solid phase ligand library (Equalizer technology)		Simple, convenient, potentially useful to discover/predict nonhuman proteins	Technology not mature and needs further development and validation
Hydrazide resins for glycopeptide-capture/thioaffinity resins for cysteinyl peptide capture		Simple, reduced proteome complexity, high sensitivity, allows for PTMs detection	Ab specificity not always quite optimal, little information provided on spatial resolution of complexed proteins, not suitable for all potential proteins in the proteome

Modified from ref. [149].

from 1, 12, or 24 h at either 4°C or room temperature, although profiles obtained from different individuals under the same conditions were consistent, probably due to metabolism of blood cells, alterations of cells' membrane integrity, and/or release of degraded products from the clot [138]. Changes in protein profile (*e.g.*, intensity of peaks; either increase or decrease) from serum samples were also observed for different clotting times at both 4°C and room temperature, probably due to either degradation of plasma peptides or formation/accumulation of new peptides during and after clotting [142, 146].

Storage conditions exert effects on plasma/serum protein profiles. Minimal changes were observed in the samples stored at room temperature within the first 4 h or 6 h, whereas noticeable changes were found after 8 h, especially for peaks in the *m/z* range < 3000 [138, 146], and were more pronounced after 24 h [147]. For plasma/serum stored up to 24 h at 4°C, the profiles were quite similar, but if time was prolonged to 48 or 96 h, significant changes were observed [146]. No major differences were found for samples stored for long term at –20°C, –80°C, or in liquid N₂ [139, 146], although freeze/thaw cycles are believed to change sample composition prob-

ably due to peptide aggregation, precipitation or adsorption to surfaces [142, 148].

3.1.4 Depletion of high abundance proteins (HAPs) and enrichment of diluted ones

Several strategies are available for depletion of HAPs from plasma/serum as illustrated in Table 3 [149]. Centrifugal centrifugation is a simple variation of membrane filtration technology where centrifugation forces a liquid against a semipermeable membrane leading to retaining solids and solutes of high molecular weight, whereas the liquid and LMW solutes pass through the membrane depending on membranes' molecular weight cut-off (MWCO) [149] that ranges from 10 to 50 kDa, and centrifugation speeds from 3000–4000 × *g* [150]. Low speed centrifugation (–4000 × *g*), use of diluted plasma/serum to a final concentration of ~5%, or use of denaturing conditions (*i.e.*, addition of ACN to 25% final concentration) [151] are required to disrupt protein–protein/peptide interactions so that LMW components that may be bound to albumin or other larger species are released and are free to pass through the membrane [152].

Different types of SPE depletion formats, including columns, cartridges, microcolumns or spin columns have

been used based on ion exchange, metal chelation, affinity ligands, dye ligands (e.g., Cibacron blue chlorotriazine ligand dye), bacterial proteins A and G, Abs (polyclonal multiple affinity removal systems, MARS), or combination of these have been marketed by companies such as Agilent, GenWay, Biotech, BioRad, Sigma–Aldrich, Amersham Biosciences, Ball Corporation, Pierce and others [84, 153, 154]. Ideally, a spin column that allows parallel processing of multiple samples in convenient microcentrifuges, and highly selective column formats that could deplete 18–22 of the most abundant proteins that compromise 98–99% of total plasma/serum protein content, but leaving LMW ones would be desirable [25].

A new MARS column from Agilent Technologies (Willmington, DE) for the specific depletion of 14 high abundant proteins from plasma/serum combined with RP-C₁₈ column for postdepleted fractionation performed at 80°C for protein recovery of immunodepleted proteins, followed by HPLC-Chip/MS analysis resulted in identification of the cytokine interleukin (IL)-27 beta chain present in pg/mL concentrations in plasma [155]. The Seppro™ Mixed 12 spin column (GenWay Biotech., San Diego, CA; now licensed to Beckman-Coulter) containing 12 avian polyclonal immunoglobulin yolk (IgY) Abs against the 12 most abundant proteins are covalently coupled to microbeads to pack the column. Recycling of the column up to 135 times did not lead to apparent loss of specificity or capacity [156]. The Sigma ProteomePrep 20 (Top 20) depletion column, which removes the 20 high abundant plasma serum proteins, allows up to 100–200-fold more liquid volume to be loaded into downstream separation and analyses after depletion. Only eight mediums to LAPs that were predominantly found in the unbound (depleted fraction) were also found in the bound fractions in 1-DE [157].

Concerns exist about the use of immunodepletion methods under nondenaturing conditions because cytokines and other low-abundance proteins bound to the target proteins may be simultaneously removed via the “albumin sponge effect”. Consequently, about 210 proteins – including some potential biomarker candidate proteins – were found to be associated with the six most abundant plasma proteins, and removal of albumin using a resin causes a significant loss of several cytokines [158]. However, the use of polyclonal antibody spin columns to deplete abundant plasma proteins has a good potential to reduce this nonspecific binding, or sponge effect [159]. Procedures that enable the recovery of low abundance proteins from immunoaffinity columns, such as use of mild solvent treatment of the bound proteins, salt-out preparation, and molecular sieve filtration [159], nonprotein binding resin [160], or membrane containing small peptides [161] to selectively remove albumin, but not albumin-bound proteins from plasma have promising potential [162]. These depletion techniques

could result in some sample dilution requiring enrichment downstream to enable depletion of LAPs [163], and the abundant proteins can themselves bind either specifically or nonspecifically to other proteins in the sample leading to their loss [164].

A recent study on human serum combining protein fractionation by Off-GEL™ IEF and RP-HPLC run and detection by an IT mass spectrometer showed that serum proteins are equally distributed at overall levels of concentration; thus, depletion columns cannot unfortunately stand their promise of making 1000s of low abundance proteins accessible by even removing the 6–20 most abundant proteins, because the next most abundant ones will have similar ratios compared to the remaining proteins as the depleted most abundant proteins have had [165].

Selective binding and enrichment of LMW peptides and proteins from human plasma using nonporous silica particles have also been described [166]. Alternative selectivity could be obtained by changing the characteristics of the nanoporous silica. Lower molecular weight proteins were best concentrated using smaller pore size silica with a LOD of 15 ng/mL for plasma spiked with insulin in a MALDI-TOF MS detector. A similar enrichment has been shown using nanoporous controlled pore glass beads [167] or nanoporous surfaces [168].

SPE disk plates for high abundance protein removal from plasma/serum samples using different functionalities such as ion exchange, dye ligand, and RP disks have employed the same principles attempted in SPE columns. The main advantage associated with the use of SPE disk plates is the increased ability for HT automation as they are generally used in a 96-well plate format allowing simultaneous processing of large number of samples robotically [169].

Precipitation of HAP from plasma/serum with simultaneous extraction of peptides and LMW proteins using organic solvents (e.g., two volume of CAN containing 0.1% TFA) in the presence of ion-pairing agents dissociates peptides and small proteins from large abundant proteins such as albumin, while small proteins and peptides stay in solution, which facilitates their analysis [149, 170].

A two step fractionation approach for plasma using MARS-6 immunodepletion column with multi-lectin affinity chromatography to deplete the glycosylated proteins allowed for identification of protein biomarkers such as angiotensinogen present at high levels in patients with obesity and its associated complications such as diabetes and hypertension [171].

The selective affinity capture of binding proteins and their complexes with downstream proteomic analysis was initially reported in the mid 1990s [172]. A number of platforms exist, which include direct immunoprecipitation [173], affinity columns, magnetic beads, biosensor

surfaces, and antibody arrays [174]. Affinity chromatography can be used not only to deplete certain types of proteins but also for enrichment of specific classes of proteins such as phosphoproteins, glycoproteins, thiosulfide proteins, ubiquitinated proteins, and characterization of groups of proteins in terms of function and structure (protein complexes) by tandem affinity purification (TAP) steps, in which a TAP tag, which consists of two type G binding domains, fused either N- or C-terminally to the target protein, and the construct is introduced into the host cell organism in order to understand the functions of protein networks. However, it must be remembered that adding tags to proteins can modulate their behavior, and the method relies upon protein over expression, which may influence signaling pathways [175].

Affinity-based techniques are advantageous if suitable reagents are available because of their high selectivity and relatively gentle elution conditions, which minimize protein denaturation [176]. N-linked glycoproteins in plasma or serum in humans or mice, respectively, could be captured using lectin affinity column (Qiagen), hydrazine resin that covalently link the N-linked glycopeptides following oxidation of the sugars [177–179], which are subsequently digested by trypsin, and N-linked tryptic peptides released from the resin by digestion with N-glycosidase, followed by LC-MS/MS [180, 181]. A number of serum proteins, including albumin, are not glycosylated and therefore are not effectively removed using these strategies [178].

Another affinity method targets cystine-containing peptides [182]. Cysteines constitute ~1.7% of amino acids in proteomes, and therefore enriching peptides with cysteines provides a substantial simplification of complex peptide mixtures. In a study on a mammary epithelial cell proteome, a thiol-specific resin was used to enrich cysteine-containing peptides before fractionation by strong cation exchange and identification by LC-MS/MS. A number of low abundance proteins were detected in the cysteinyl-enriched fraction that was not identified in non-enriched fractions [183].

Protein phosphorylation is one of the most common PTMs that regulate protein localization, complex formation and degradation. The most common methods for enrichment of phosphoprotein samples involve either affinity chromatography using immobilized antiphosphopeptide Abs, IMAC with immobilized Fe^{3+} , Ga^{3+} , Al^{3+} , or Zr^{4+} ions [184], or the use of titanium dioxide microcolumns followed by downstream MS analysis. These supports are now available in a range of formats including ZipTips (Millipore, Billerica, MA), SwellGel Gallium disks, and as part of a CD microlaboratory (Gyrolab MALDI IMAC1 (Uppsala, Sweden)). These techniques have been successfully used to identify numerous new phosphoproteins and phosphorylation sites [185–187]. In some cases, initial enrichment using antiphosphotyrosine Abs,

which have better specificity than the antiphosphoserine/threonine Abs, followed by IMAC have been used [188]. Although generally stable, in one study a batch-to-batch variation of C_8 magnetic particles has been observed [142].

Paramagnetic nonporous particles with chromatographic functionality have gained acceptance as platforms for the chromatographic manipulation of samples prior to downstream analysis [189]. Such beads are commercially available (Dyna, CPG, Bruker Daltonics, Miltenyi, Polymer Labs, Promega, QuickPick, BIAcore, IAsys, Vir, BioRad) in a wide range of functionalities including biological affinity, IMAC (Cu and Fe), RP (C_3 , C_8 , and C_{18}), anion- and cation-exchange. They offer a number of advantages, making them ideally suited to sample preparation for downstream proteomic analysis, as the process is simple and gentle, often allowing protein complexes to be recovered intact [189]. The magnetic particles can subsequently be recovered by such devices as the Kingfisher Magnetic Particle Processor (Thermo Scientific, Waltham, MA) or a Tecan Genesis Liquid handling workstation (Tecan, Durham, NC) [190–192]. The magnetic beads technology has allowed for the development of automatic magnetic particle based immunoassay systems (e.g., Beckman-Coulter Access (Fullerton, CA), Bayer ADVIA Centaur (Bayer, Leverkusen, Germany), Abbott ARCHITECT i2000 (Abbott Diagnostics, Abbott Park, IL)) [193].

Biosensor technology (e.g., BIAcore, Uppsala, Sweden; IAsys (Neosensors, Sedgefield, UK)), used as affinity detector combined with micropreparative HPLC, has been used for the identification, purification, and characterization of ligands for orphan receptors during chromatographic identification of the ligands of interest. Biosensors were also used as microaffinity purification platforms (both flow-based and cuvette-based systems) [194–196] for subsequent capture and MS/MS peptide analysis.

Mass spectrometric immunoassay (MSIA) that couples mass identification of proteins with antibody capture results in a unique biomarker signature, which overcomes the inability of traditional ELSA to recognize the specific form of the ligand assayed in spite of its excellent sensitivity [67, 197]. For example, whereas the performance of 115 Ab-antigen pairs was investigated in a study aimed at developing protein microarrays, only 20% of the arrayed Abs provided specific and accurate measurement of their cognate ligands at concentrations at or below 1.6 $\mu\text{g/mL}$ [198]. Immuno-MS has been used to analyze a number of potential biomarkers in urine (e.g., β -2 microglobulin, transthyrein, cystatin C, urine protein 1, retinol binding protein, albumin, transferring, and human neutrophil defensin peptide) using individual microcolumn tips (MSIA Tips, Intrinsic Bioprobes, Tempe, AZ) loaded into a multichannel pipette [199]. Rapid and cheap HT production of mouse-derived mAb using immunization, automated fusion and cell culture,

Table 4. Chromatographic selectivities for multidimensional separation/fractionation methods based on physical or chemical properties

Method	Properties	Elution conditions
Anion/cation exchange Chromatofocusing RP	Charge Charge Hydrophobicity	Above/below pI, increasing salt concentration pH gradient Step wise or gradient elution with increase in concentration of organic solvent
Hydrophobic/hydrophilic Interaction Size exclusion	Hydrophobicity Size (Stoke's radius)	Step wise or gradient elution with increase in organic solvent concentration/decreasing salt concentration Aqueous buffers often with low levels of detergents to minimize nonspecific adsorption
Affinity enrichment/depletion Lectin chromatography Metal affinity	Affinity Affinity Affinity	Competition, conformational change Competition, e.g., glucosamine Competition using increasing imidazole concentration or pH change
Ligand dye HAP	Pseudo affinity Pseudo affinity	Change in pH, salt concentration or use of a displacer Increasing phosphate concentration for this mixed-mode ion exchange

Modified from refs. [67] and [204].

and sensitive screening based on antigen-coated microarrays has been reported [198]. Such Abs need to be characterized for affinity and specificity using a technology as immuno-MS. These developments would ultimately lead to panels of highly specific Abs raised against all proteins predicted in the genome [84].

By optimizing a magnetic bead-based platform (Protein G beads linked with immobilized polyclonal Abs) amenable to HT peptide enrichment, followed by multiple reaction monitoring (MRM) MS using a stable isotope standards with capture by anti-peptides Abs (SISCAPA) and enrichment measured by selection ion monitoring, which employed a linear IT mass spectrometer for quantitative testing of biomarker candidates, an ion signal enhancement of $>10^3$ was achieved, believed sufficient for quantifying biomarkers in plasma at the range of ng/mL, which are applicable to any protein and biological fluid of interest [200].

Use of protein equalizer technology to reduce protein concentration differences by sharply reducing the differences of the most abundant components, while simultaneously enhancing the concentration of the most dilute species (*i.e.*, equalize) body fluid samples such as plasma/serum or urine. The technology uses a diverse solid phase library of combinational peptide affinity ligands coupled to spherical porous beads $\sim 65\ \mu\text{m}$ in diameter (Equalizer™ beads) that carry 50 pmol of hexapeptides supplied by Ciphergen Biosystem, Fremont, CA, which incorporate 20 different amino acids for synthesis of different ligand structure [201]. The progressive increase in detectable species when using larger sample/bead ratio suggests that theoretically 20^6 or 64 million protein species can be enriched [202]. Because the library has equal amounts of each ligand, theoretically the maximum amount of each protein binds the same number of

ligands. Under overloading condition, this has the effect of diluting those proteins present in excess of the ligand concentration, and concurrently those of relatively lower abundance. Unbound components are washed out, and capture species are desorbed [203]. The entire sample treatment takes about half a day and yields a protein solution that could be measured by MS. This fractionation method under the simplest condition (adsorption followed by a single elution), or with sequential desorption, or in association with other fractionation methods, can theoretically be valuable in situations when proteins are predicted by genome or mRNA sequences, but previously undetected, as in biological extracts of nonsequenced organisms, and they may now – at least theoretically – be detected [203].

Analysis of serum passed through a solid phase ligand library and sequentially eluted showed altered patterns compared with 2-DE; however, the new protein spots were not sequenced [202]. Therefore, it is not clear if solid phase ligand libraries substantially increase the detection of low abundance plasma proteins, nor is it clear if such strategies can be used in the near future to discover quantitative changes in different plasma samples [157]. Further, developments of this technique are thus warranted before its utility for biomarker discovery can be fully evaluated.

Chromatographic selectivities for multidimensional separation methods based on physical or chemical properties are presented in Table 4.

4 Sample fractionation to reduce proteome complexity

Due to the high complexity of plasma/serum samples, various fractionation methods have been used either to

Table 5. Common advantages and limitations of separation methods for mass spectrometers

Ionization source	Advantages	Disadvantages
Surface-enhanced SELDI chips	Affinity capture on MALDI chips with chromatographic functionality, ease of use, automation, convenience, low sample volume, raw samples analyzed, various chip surfaces, does not require sophisticated bioinformatics tools, detection with a broad molecular mass region in a single analysis	Loss of important information, problems at preanalytical and postanalytical steps, bias toward high abundant proteins particularly in the low mass range, performance could change over time
Derivatized carrier material (MELDI)	Allows for detection of larger number of peptides than SELDI due to use of particles with higher surface areas, permits raw sample analysis, HT	Carrier material must be carefully chosen, only highly porous, spherical and low micrometer size range particles can be used as carriers
Surface-derivatized magnetic beads	Can be automated, employs wide range of derivatized beads with different functional groups, sensitive, compatible with MS	Lack of reproducibility between commercial batches of the same beads
Glycoprotein/glycopeptide capture	High selectivity, reduced sample complexity	Loss of information on nonglycosylated proteins, increased false positive protein identification
Nonporous substrates (silicon wafers, silica particles and glass beads)	Allows for harvesting of distinct subsets of the proteome	Not a mature technology, needs standardization/validation
2-DE chromatography	Applicable to large molecules, high resolution, allows visualizing changes in molecular mass (M_r), pI or PTMs	Not applicable to peptides Is or M_s poorly represented
CE	Automation, relatively sensitive, low sample volume needed, low cost, MS/MS compatibility	Not well suited for peptides >20 kDa, precipitation of peptides in capillaries when acidic running buffers are used
LC	Automation, highly sensitive, accurate, multidimensional, versatile, HT potential, MS/MS compatibility	Time consuming, sensitive toward interfering compounds, limited mass range, often unsuitable for analysis of intact proteins
Protein microarrays	HT, low sample volume, chips have potential for assaying a wide range of biochemical activity, various platforms and detection methods are available	Abs are not available for all screened proteins, no standardization is available for biomarker discovery, low sensitivity, qualitative

Modified from refs. [25, 84, 129, 135].

generate several fractions (e.g., gel, CE, and LC), or to selectively obtain a particular subset of proteins/peptides with common features based on their similar affinities to a particular solid support (e.g., SELDI, MELDI, surface-captured magnetic beads, nonporous substrates) [149]. These strategies are illustrated in Table 5.

A higher dimensional 4-D separation strategy for an in-depth analysis of plasma/serum proteome that combines three orthogonal protein separation methods (protein depletion by affinity column, microscale solution IEF using ZOOM fractionator, and 1-DE), slicing gels and in-gel tryptic digestion carried out on sliced gels, followed by nano LC fractionation and MS/MS analysis, is believed to allow a detection dynamic range approaching 10^9 (<10 ng/mL), which is the concentration range expected for biomarkers in blood [157].

Heparin chromatography has been used to fractionate proteins from extracts of prokaryotic organisms or eukaryotic cells. Heparins are negatively charged polydispersed linear polysaccharides that have the ability to bind a wide range of biomolecules including enzymes, serine protease inhibitors, growth factors, extracellular matrix proteins, DNA modification enzymes and hormone receptors. In this chromatography, heparin is not only an affinity ligand but also an ion exchanger with high charge density and distribution, in which biomolecules can be specifically and reversibly adsorbed by heparins immobilized on an insoluble support, having the advantage of being able to enrich heparin-binding proteins using its concentration effect, which is particularly advantageous for analysis in 2-D, MS or other proteomics approaches [205].

IMAC, a useful fractionation method used to enrich metal-associated proteins, represents an affinity separation approach based on the interaction between proteins and metal ions immobilized on a solid support. By changing various metal ions and other experimental conditions such as pH and elution composition, IMAC can selectively isolate metal-binding protein fractions for further specific proteomic analysis, as characterizing the metalloproteome and its PTMs [206].

Other nonchromatographic purification have also been developed that can deal with protein suspensions [207]. A three phase partitioning (TPP) was originally described for interfacial precipitation of proteins, as well as for protein refolding. As such, TPP lacks selectivity, but was adequate for preparation with reasonable purity [208]. A recent version known as macroaffinity ligand facilitated TPP (MLFTPP) using eudragit S-100 as the affinity microligand, xylanase enzyme was purified from crude mixture of the fungus *Aspergillus niger* protein [209]. An aqueous two-phase system (ATPS) (e.g., PEG and dextran or phosphate) could partition introduced proteins forming two phases. Separation could be achieved by manipulating the partition coefficient of proteins by varying the average molecular weights of the polymers, the large strength of the salts or introducing an affinity ligand [210]. Expanded bed chromatography (EBC) is a technique that utilizes all the concepts of traditional backed bed technology, but the bed (i.e., calcium alginate or zinc alginate beads) is a fluidized form [211]. It combines filtration/centrifugation, concentration, and purification in a single step. EBC has benefited from using an immobilized affinity medium that can fluidize such as polyhistidine fusion tags [212].

5 Sample preparations from frozen tissue samples

The local concentration of the biomarker is expected to be high in the vicinity of the tumor microenvironment. Therefore, fine-needle aspiration biopsies (FNABs) are one way to obtain these samples [134]. Because many different cell types are typically present in tissue biopsies, laser microdissection (LMD) techniques have been developed to provide a rapid method for separating and processing homogenous subpopulation of cells for biochemical analysis [213]. Use of LMD may subject samples to potential artifactual processing, including changes at two different stages: (a) during the stage of tissue sections that enables selection of the relevant cell types, and (b) during the dissection process itself. These changes could impact the level of protein recovery and the quality of subsequent proteomic studies [214]. Isolated cells and captured minute tissue samples can then be directly analyzed employing, for example, MALDI-MS, or through the use of an automated multidimensional HT separation platform

that combines CIEF, with nano-RP LC [215]. CIEF is a variant of the commonest mode of CE (CZE) that combines the high resolution power of conventional gel IEF with CE instrumentation, and because of its focusing effect, it has often been used as the first step in multidimensional separation of complex mixtures of peptides and proteins [216] based on *pI* and a running buffer that contains ampholytes generating an electrically stable pH gradient, thus providing the highest possible efficiency for protein separation. The high analyte concentrations in small peak volumes as a result of electrokinetic focusing/stacking and the resolving multidimensional separation results in sensitive proteome analysis by enhancing the dynamic response and detection sensitivity of the coupled MS instruments. Instead of performing multi-runs or multidimensional separations, comparable or even better HT proteome results could theoretically be achieved by simply increasing the number of CIEF fractions due to the intrinsic high resolution nature of electrokinetic focusing, a feature that is particularly important for proteome analysis of limited tissue samples [217].

6 Dealing with formalin-fixed, paraffin-embedded (FFPE) tissue

FFPE tissue is the most common clinical specimen available after fixing and paraffin wax embedding for every tissue, from biopsy or surgical origin samples, for application of diagnostic assays on tissue after microscopic examination, and this huge amount of human material becomes a valuable resource for research in molecular medicine and biomarker discovery. It has been shown that protein crosslinking (and also nucleic acid) [218], due to formalin fixation, prevents protein profiling by Western blot and protein microarrays [219, 220]. The only routinely employed method currently available for protein analysis in FFPE tissues is immunohistochemistry, which is a qualitative technique. Although, a wealth of information exists on the expression signature of mRNA expressed by a specific type of cancer [221, 222], little data are currently available about protein expression signature in normal and cancerous cells [223–226] because difficulties of obtaining clinical samples, as well as the complexity of the dynamic proteome.

A shotgun proteomic method called direct tissue proteomics (DTP) was developed to provide extraction procedure that disrupts the crosslinked proteins from FFPE tissue samples, allowing for chemical identity of proteins in cells, tissues and fluids by MS/MS analysis [225]. Different extraction buffers were tested. ACN buffer (30% ACN, 100 mM ammonium carbonate), which is compatible with trypsin digestion and direct LC-MS/MS analysis can extract from 13 to 42% of total extractable proteins of large quantities of available samples. Another buffer (buffer D) was recommended; it contains radio-immunopreci-

pitiation (RIPA) buffer (150 mM NaCl, 10 mM Tris-HCl (pH 7.2), 2% SDS, 1% Triton X-100, 5 mM EDTA) heated at 94°C for 30 min followed by 60°C for 3 h to rehydrate proteins and hydrolyze the formaldehyde crosslink, followed by incubation with 1 µg of trypsin in buffer D (1:20 dilution) for 18 h at 37°C, then sample lyophilization and resuspension in buffer B (5% CN, 0.5% acetic acid, 0.005% heptafluorobutyric acid) and analyzed on a Finnigan LTQ-LIT mass spectrometer coupled to nano electrospray source. A 0.1% RapiGest buffer (Waters, Milford, MA) in 50 mM NH₄HCO₃, which is also compatible with MS, was found to give ~77% higher protein content than buffer D by densitometric quantification of 2-D silver stained gels [225].

Because DTP method is not quantitative, an absolute quantification method termed AQUA that employs an internal peptide standard was used for quantification of pictogram levels of prostate-specific antigen (PSA) in normal and cancerous prostate FFPE tissue. Using minute prostate biopsy sections, 428 prostate-expressed proteins were identified. The DTP strategy is a general method that is believed to provide a roadmap for successful identification of critical molecular targets of multiple cancer types [227].

A commercial multiplexed protein extraction system that is robust, fast, standardized and easy to use as a protein measurement technique for the solubilization of nondegraded, full length, and immunoreactive proteins from FFPE tissue samples was developed. It is specifically designed for the solubilization of high amounts of proteins from 10% neutral formalin-fixed tissue samples (Qproteome FFPE Tissue Kit, Qiagen) from cancer and noncancer tissues like colon carcinoma/non cancerous colon tissue, gastric cancers/nontumor gastric tissue, breast cancer, and nontumorous pancreatic tissue was employed. After deparaffination of cut sections placed on slides, with other unfixed frozen cryostat sections for comparison, it was possible to conveniently detect membrane, cytoplasmic and nuclear proteins, and no differences were found in the protein yield and abundance by Western blot and RP protein microarrays. Extraction of protein membrane (as demonstrated by the analysis of Her2 and E-cadherin proteins immunohistologically) in breast cancer biopsy was employed to test that technique [228]; which was found to be practical.

7 Concluding remarks and future prospects

It is evident from the body of this review that the field of sample preparation for proteomics is still in its infancy. There is no general standardized strategy for overall sample preparation, separation or purification. An ideal strategy, of course, is to entirely avoid sample preparation, but unfortunately today mass spectrometers do not

allow for direct identification/quantification of myriad proteins present in complex biological matrices. Until that goal is realized, a HT preparation strategy, with application of microfluidic devices, microchips (lab-on-chip) and automation, focusing on isolation of protein from organelles, as well as analysis of PTMs such as phosphopeptides, glycosylated proteins and other PTMs that play important roles in signal transduction processes is envisioned, in order to have a better understanding of the global processes occurring within cells from which sample preparation should be mild and suitable for extraction of noncovalent complexes between proteins, peptides, nucleic acids, and metabolic products [8, 13].

Major drawbacks to the general acceptance of multidimensional HPLC purification strategies are their being technically demanding, time consuming, need to be optimized for recovery and reproducibility, not easy to automate for HT analysis and requirement for extensive MS analysis time that could result in data analysis bottlenecks. Alternative platforms such as magnetic beads, multiwell plates or chips could address many of these issues. Simple batch wise elution techniques can lead to effective fractionation that is amenable to a HT automated application. Use of multidimensional techniques for MS analysis can reduce sample complexity and also increase the number of samples analyzed [176]. Improvement in data processing and analysis by integrating these processes into a linear process will increase overall efficiency [229].

The discovery role of potential biomarkers and drug targets far exceed the rate of early validation, and the gap is expected to increase [135]. Therefore, standardization of sample preparation methods to obtain reproducible data between laboratories is a must. Moreover, more investigation is needed focusing on reduction of sample complexity, developing promising sample preparation methods such as improved multiaffinity removal system, multidimensional LC, and use of nonporous solid phases [149]. Subcellular fractionation allows access to intracellular organelles and multiprotein complexes; LAPs and signaling complexes can be enriched, and at the same time, the complexity of the sample can be reduced [90].

Fractionation of protein mixtures to isolate species by their common biological activity is an approach that is not yet well established, not because of lack of interest, but rather because of lack of effective methods for immediate implementation. Selection of protease activities would no doubt have a strong impact on the understanding of specific pathway regulations with direct interest in diagnostic. Most proteases are part of very low abundance species that might stay silent for long periods, but can be detected by their specific peptide signature [65].

Application of SELDI technology and the generated proteomic patterns for the analysis of differences in proteins between healthy and cancer-bearing individuals

has raised many concerns due to various discrepancies, as illustrated in the body of this review, in a recent review on the subject [230] and in Table 5 [231, 232]. MALDI profiling, although has given better results than SELDI, still needs further standardization and evaluation [233]. Although, a recently developed carrier-based approach called MELDI is an improvement on the MALDI approach, and appears to enhance the robustness and throughput of large-scale proteomic studies [234, 235], nevertheless, it still needs confirmation [230].

The development of orthogonal high-dimensional proteomic strategies that include two or more protein separations has been shown to overcome to some extent the complexity of the plasma proteome leading to the detection of a large number of LAPs (<100 ng/mL), where cancer biomarkers are expected to be found, and also the identification of their PTMs when using intact protein fractionation schema together with shotgun LC-MS/MS analysis methods that are associated with the disease; this is a feature that is not readily available with regular protein digest-based fractionation approaches [136].

With an increased use of unique separation platforms, together with an equally expanding repertoire of fractionation technology aiming at precise molecular characterization of chemical modifications contributing to the complexity of protein patterns, coupled with quantitative and differential methods for protein analysis by MS, exceptional target protein biomarker signatures for diagnostic, prognostic, and evaluating response to therapy, which are more relevant than current descriptive or nucleic acids-based biomarker technology, is expected to be attained in the not too distant future.

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