INTACT-PROTEIN BASED SAMPLE PREPARATION STRATEGIES FOR PROTEOME ANALYSIS IN COMBINATION WITH MASS SPECTROMETRY

Hong Wang and Sam Hanash*

Department of Pediatrics, University of Michigan, Ann Arbor, Michigan 48109-0656

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The complexity of tissue and cell proteomes and the vast dynamic range of protein abundance present a formidable challenge for analysis that no one analytical technique can overcome. As a result, there is a need to integrate technologies to achieve the high-resolution and high-sensitivity analysis of complex biological samples. The combined technologies of separation science and biological mass spectrometry (Bio-MS) are the current workhorse in proteomics, and are continuing to evolve to meet the needs for high sensitivity and high throughput. They are relied upon for protein quantification, identification, and analysis of post-translational modifications (PTMs). The standard technique of two dimensional poly-acrylamide gel electrophoresis (2D PAGE) offers relatively limited resolution and sensitivity for the simultaneous analysis of all cellular proteins, with only the most highly abundant proteins detectable in whole cell or tissue-derived samples. Hence, many alternative strategies are being explored. Numerous sample preparation procedures are currently available to reduce sample complexity and to increase the detectability of low-abundance proteins. Maintaining proteins intact during sample preparation has important advantages compared with strategies that digest proteins at an early step. These strategies include the ability to quantitate and recover proteins, and the assessment of PTMs. A review of current intact protein-based strategies for protein sample preparation prior to mass spectrometry (MS) is presented in the context of biomedically driven applications. © 2004 Wiley Periodicals, Inc., Mass Spec Rev 24:413–426, 2005

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I. INTRODUCTION

The sequencing of the human and other important genomes has opened the door for proteomics by providing a sequence-based framework for mining the proteome of healthy and diseased cells and tissues (Chalmers & Gaskell, 2000; Mann, Hendrickson, & Pandey, 2001; Pasa-Tolic et al., 2002; Yarmush & Jayaraman, 2002; Aebersold & Mann, 2003; Bauer & Kuster, 2003; Hanash, 2003; Lin, Tabb, & Yates, 2003). Major applications of proteomics include: (1) expression profiling to determine the identity, abundance, modification state, and sub-cellular localization of proteins, all of which are context-dependent; (2) determination of protein-interaction networks; and (3) elucidation of protein structure. With the emergence of soft ionization techniques such as fast atom bombardment (FAB), matrix-assisted laser desorption ionization (MALDI), and electrospray ionization (ESI) more than a decade ago (Barber et al., 1981; Karas & Hillenkamp, 1988; Fenn et al., 1989), biological mass spectrometry (Bio-MS) has become a standard tool for protein analysis. Biological samples subjected to proteomic analysis consist of three major types: (1) tissues, (2) cell populations, and (3) biological fluids. A common feature of biological samples is their extraordinary complexity because of the high multi-dimensionality of their protein constituents, which differ in their cellular and subcellular distribution; their occurrence in complexes; their charge, molecular mass, and hydrophobicity; and their expressed level and their post-translational modification (PTM). It is, therefore, unrealistic that any one analytical technique would be well suited to deal with all the protein complexities. As a result, various schemes are currently being implemented to reduce the complexity of biological samples prior to analysis by mass spectrometry (MS). Desirable objectives include extending the detection, quantification, and identification to low-abundance proteins, assessment of protein distribution among cells, and subcellular structures and assessment of their PTM.

Innovations in MS continue to have a substantial impact on proteomics. Nano-electrospray techniques (Wilm & Mann, 1996; Shevchenko et al., 1997) combined with a hybrid quadrupole time-of-flight mass spectrometer tandem mass analyzer (ESI Q-TOF MSMS) enable extensive fragmentations to produce collision-induced dissociation (CID) spectra that allow unambiguous protein identification by peptide sequence tags through protein sequence database searches. High-throughput proteomic analysis may also be performed with a MALDI Q-TOF MSMS tandem instrument (Loboda et al., 2000; Shevchenko et al., 2000) and MALDI-TOF-TOF MSMS tandem MS (Medzihradszky et al., 2000). A new ion source for Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS) enables quick changes between MALDI and ESI modes (Baykut et al., 2002). A new concept of a sample inlet technique, microfabricated fluidic, and array systems have been coupled with MS

**Correspondence to:* Sam Hanash, Department of Pediatrics, University of Michigan, 1150 West Medical Center Drive, MSRB 1, Room A520, Ann Arbor, MI 48109-0656. E-mail: shanash@med.umich.edu

for protein analysis (Figeys & Pinto, 2001; Figeys, 2002). All of the recent innovations notwithstanding, MS techniques are still most effective when applied to samples of limited complexity. Thus, analyzes of whole tissue and cell proteomes, with the vast dynamic range of their protein abundance and the occurrence of multiple protein isoforms, present a major challenge for MS. As a result, only a limited repertoire of proteins and peptides is uncovered. That limitation puts substantial emphasis on sample preparation to reduce complexity through sample fractionation to allow a more comprehensive analysis of constituent proteins. Figure 1 shows the flow of intact protein-based preparation strategies used to reduce sample complexity and to enhance overall sensitivity prior to Bio-MS.

II. CURRENT STATUS OF TWO DIMENSIONAL PROTEIN-SEPARATION SYSTEMS

A. Two Dimensional Gel-Based Separations

Some three decades ago, two dimensional poly-acrylamide gel electrophoresis (2D PAGE) emerged as a separation technique that is capable of resolving thousands of cellular proteins in a single gel. It was idealized that 2D gel systems could display all cellular protein constituents. However, it became clear that the several thousand cellular proteins that may be displayed in a typical 2D gel of a tissue or cell lysate represented a relatively small proportion of the totality of the proteins expressed. This limit is because many of the proteins detectable in 2D gels of whole-cell lysates represent multiply modified forms of a limited numbers of proteins. Thus, 2D PAGE of whole cell or tissue lysates allows an analysis of a limited repertoire of cellular proteins that represent mostly abundant cytosolic proteins. Intrinsic limitations of 2D PAGE tend to exclude highly hydrophobic membrane proteins, highly acidic or basic proteins, and low-abundance proteins. Thus, the hopes of displaying all cellular proteins in a 2D gel have not materialized. However, over the past quarter century, several innovations have been explored to improve the utility of 2D gels.

To improve the yield of low-abundance proteins in 2D gels, various schemes have been implemented for sample preparation prior to 2D gel analysis. Liquid-phase isoelectric focusing (IEF) has been utilized to pre-fractionate, in a non-gel medium, complex based on the p*I* of the individual proteins. Herbert & Righetti (2000) proposed a protein sample pre-fractionation approach to isolate proteins into several groups according to the

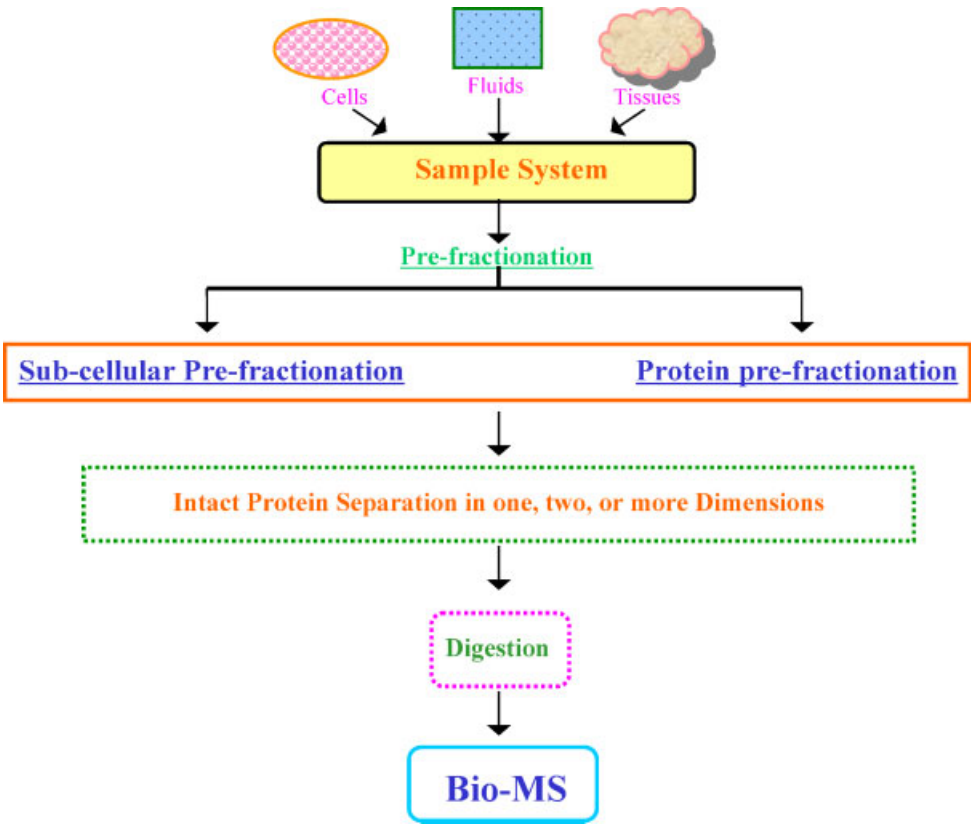

FIGURE 1. A flow chart of intact protein-based sample preparation strategies for biological mass spectrometry (Bio-MS) analysis in proteomics. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

p*I* within multi-compartment electrolyzers (MCE) that are delimited by immobilized isoelectric membranes with pH values of 3.0, 4.0, 5.0, 6.0, and 10.5. They applied this liquid-phase IEF method to pre-fractionate *Escherichia coli* whole-cell extracts and human plasma. Proteins in each fraction were subsequently separated by narrow-pH range 2D PAGE. In plasma separations because albumin was concentrated within the membranes between pH 5.6 and 6.1, the acidic and basic chambers were both free of albumin; that method resulted in an increase in the number of highly acidic and basic proteins in the fractionated sample compared to whole plasma. Pedersen applied the same technology to fractionate alkaline proteins from *Saccharomyes cerevisiae* solubilized-membrane protein mixtures within the MCE compartment between pH 7.5 and 10.5. The concentrated alkaline fraction was subjected to narrow-pH range 2D PAGE followed by MALDI-TOF MS (Pedersen et al., 2003). A total of 93 unique proteins were identified in this pH 7–10.5 fraction, including 30 low-abundance proteins with the codon adaptation index (CAI) below 0.2, 20 integral membrane proteins, and 10 membrane-associated proteins.

Zuo and Speicher developed a microscale solution IEF (μsol-IEF) device that consisted of six to seven separation chambers bound by the immobilized isoelectric membranes to pre-fractionate mouse serum into a series of well-defined pools prior to subsequent analysis with 2D PAGE (Zuo et al., 2002). After IEF, each chamber contained only proteins with a p*I* between the pH of the boundary membranes of that chamber. That pre-fractionation method fractionated complex protein samples into very narrow ranges (<0.5 pH units) with an enhanced ability to analyze low-abundance proteins. Six- to 30-fold greater protein loads were practical for non-albumin fractions in the subsequent narrow-pH range 2D gels, and that in turn increased the dynamic range of protein analysis. That method was also used to pre-fractionate the whole-cell extract of a human breast-cancer cell line into seven discrete pools, including four sequential 0.5 pH range fractions in the pH 4.5–6.5 region, which contained the majority of cellular proteins (Zuo & Speicher, 2002). These four pH pools (0.5 pH units) were applied onto the narrow pH range gels for further separation.

Alternatively, liquid-phase IEF in the Rotofor system (Bio-Rad Laboratory) with 20 IEF cells has been utilized to pre-fractionate human cerebrospinal fluid (CSF) prior to 2D PAGE (Davidsson et al., 2002). Proteins in selected IEF fractions were further resolved on SYPRO-Ruby-stained 2D PAGE gels. It was found that more protein spots were detected in 2D gels from pre-fractionated CSF compared with direct 2D PAGE separations of CSF. Some low-abundance proteins, including cystatin C, IgM-kappa, β-2 microglobulin, alpha-1-acid glycoprotein, acetyl-coenzyme A carboxylase-alpha, and hemopexin, were identified in pre-fractionated but not in non-fractionated CSF. Low-abundance forms of post-translationally modified proteins, as in the case of alpha-1-acid glycoprotein and alpha-2-HS glycoprotein, could be enriched, thus improving overall resolution and sensitivity. Similarly, Zuo et al. (2002) utilized an IEF method to fractionate human-breast cancer-cell lysates prior to 2D PAGE, with improved results.

Görg et al. (2002) revisited the use of flat-bed IEF in granulated Sephadex gels, namely as a pre-fractionation procedure that was applied to mouse-liver proteins. Ten gel fractions were simply extracted with a spatula, and each fraction was directly applied onto the surface of corresponding narrow-range IPG strips and subjected to further separation by 2D PAGE. Proteins in the Sephadex gel fractions migrated electrophoretically onto the IPG gel with high efficiency and without any sample dilution. One milligram of mouse-liver proteins was pre-fractionated in this fashion, and neither protein precipitation nor horizontal or vertical streaking was observed in the subsequent narrow pH range 2D gels. As a result, the considerably greater numbers of protein spots detectable in 2D gels indicated enrichment in low abundance proteins.

These improvements in resolution and sensitivity are promising, and stem from the ability to apply greater amounts of proteins. The gains are obviously achieved at a certain cost-namely, that the rather complicated procedure of 2D gels is made even more complicate from the need to integrate data and images across multiple 2D gels. There is a need for a critical assessment of reproducibility with such schemes as well as an assessment of the extent to which certain proteins may be subjected to modifications that could have a negative impact on sample analysis.

B. Two Dimensional Liquid-Based Separations

There is a great deal of interest at the present time in developing gel-free systems for protein analysis because of their potential for multiplexing (Liu et al., 2002; Wang & Hanash, 2003). An analogy may be made to DNA sequencing, notably as utilized in the genome project, which received a considerable boost when the switch from gel-based approaches to a gel-free technology took place. Multi-modular combinations of HPLC, liquid-phase IEF, and capillary electrophoresis (CE) provide various options to develop high-resolution orthogonal 2D liquid phase-based strategies for the separation of complex mixtures of proteins. Such strategies include size-exclusion chromatography (SEC)–CE or SEC–reversed-phase liquid chromatography (RPLC) was used by Jorgenson's group to fractionate protein mixtures in *Escherichia coli* lysates (Larmann et al., 1993; Opiteck et al., 1998). Le Coutre et al. (2000) analyzed *Escherichia coli* membrane proteins with affinity chromatography, followed by on-line RPLC–MS. Feng et al. (2001) reported the use of ion-exchange chromatography (IEC) followed by on-line eight-channel parallel RPLC–ESI-MS to purify recombinant proteins in a high-throughput fashion. A major advantage of liquid separations is that proteins are maintained in solution that allows on-line intact protein characterization by MS as well as protein recovery. Our group developed a novel 2D IEF-RPLC system to fractionate or resolve large numbers of cellular proteins. These protein fractions were recovered and applied to protein biochips to determine their antigenicity in cancer (Wall et al., 2000; Madoz-Gurpide et al., 2001; Wang & Hanash, 2003). The capacity of the 2D separation system in practice is limited to resolving no more than 10,000 protein forms according to Giddings' model, if each dimension has a capacity of 100; that capacity may not be sufficient to achieve complete resolution of a cell or tissue proteome. It is, therefore, beneficial to reduce sample complexity as much as possible.

III. STRATEGIES TO REDUCE SAMPLE COMPLEXITY BY ENRICHMENT FOR SPECIFIC CELL POPULATIONS AND SUB-CELLULAR STRUCTURES

A. Reducing Cellular Heterogeneity

The heterogeneous nature of tissue samples makes their direct proteomic analysis difficult, particularly when the cell type of interest is under-represented in the sample. A traditional way to reduce cellular heterogeneity prior to analysis of tissue samples is to disaggregate the tissue by treatment with collagenase or by other means, followed by a separate analysis of specific cell populations of interest. This traditional approach is still widely relied upon, particularly when large numbers of cells are needed. An elegant approach to reduce cellular heterogeneity and to extract a particular cell population in a tissue is laser capture microdissection (LCM), a technology that was developed at the National Cancer Institute (Emmert-Buck et al., 1996). It has been successfully used to isolate single cells within a tissue section (Emmert-Buck et al., 1996). Cells can be selected according to their phenotypic and functional characteristics. The major limitation of the LCM approach for proteomics is the labor-intensive nature to extract a sufficient number of cells for proteomic analysis.

Palmer-Toy et al. (2000) described a rapid and sensitive method to obtain an abridged protein-expression profile from microdissected human-breast tissue cells by direct acquisition of MALDI-TOF mass spectra from LCM transfer films. Four cell populations, including normal stromal cells, normal epithelial cells, ductal carcinoma *in situ*, and invasive ductal carcinoma, were isolated from a single frozen section of human breast by LCM, and were subjected to direct MALDI-TOF analysis. Distinct mass spectra were obtained from 1,250 cells from each of the four cell types. The stromal cells revealed several prominent peaks in the 4.5–7.0 kDa range. Those peaks were attenuated or absent in the spectra from cells of epithelial derivation. A series of high-mass peaks from 45 to 60 kDa distinguished the invasive carcinoma spectrum from that of normal epithelium and from the stromal spectrum as well. Xu et al. (2002) reported a comparison of mass spectra obtained from human breast tissue that contained invasive mammary carcinoma and normal breast epithelium, using LCM MALDI-TOF MS. More than 40 peaks were identified that significantly differed in intensity between invasive mammary carcinoma and normal breast epithelium. Bhattacharya used LCM to procure cancer cells from archived human lung tissue that contained an adeno-carcinoma and squamous cell carcinoma. The captured cancer cells were mixed with matrix solution, and that solution was deposited on the MALDI target for direct MALDI-TOF MS analysis (Bhattacharya, Gal, & Murray, 2003). The results showed that half of the observed peaks in the mass range between 1,000 and 4,000 Da were indicative of either adenocarcinoma or squamous cell carcinoma, and may be used as a fingerprint for those cancer types.

Lawrie et al. (2001) used LCM to selectively microdissect tumor cells from colon cancer tissues. The protein mixtures recovered from the captured cells were separated and characterized by 2D PAGE/Bio-MS. A method that utilized an adjacent stained section to guide the dissection of an unstained region of interest was used to overcome problems that resulted from an immuno-histochemical marking of the dissected cells; that staining has detrimental effects on protein analysis by 2D PAGE (Wong et al., 2000). Moulédous et al. applied this approach to unstained rat brain tissues to precisely dissect nuclei from specifically defined brain regions. Proteins from the captured tissue cells were extracted and separated by 2D PAGE, and selected spots were identified by Bio-MS (Moulédous et al., 2003).

A potentially spectacular way to reduce tissue heterogeneity is direct, *in situ*, mass spectrometric analysis of cellular constituents of a tissue—as pioneered by Caprioli's group. With this approach, imaging MS is undertaken for the analysis of polypeptide expression in tissue sections, where the spatial array of specific polypeptides present in neighboring cells is profiled by MALDI-TOF MS (Caprioli, Farmer, & Gile, 1997; Stoeckli et al., 2001). Imaging mass spectra for different cell populations of interest are determined and are compared with each other and between healthy and disease tissues. In a study of human glioblastoma, tumor cells displayed many protein differences compared to normal tissue. For example, a protein of molecular mass 4,964 Da was localized to the outer area of tumors and was identified as thymosin β4 (Tβ4), an immuno-regulatory peptide that has ability to sequester cytoplasmic monomeric actin. This concept offers the tantalizing prospect that imaging MS may be used, for example, intra-operatively to assess the surgical margins of excised tumors (Chaurand, Schwartz, & Caprioli, 2002). The major limitation of this approach, as with other approaches for direct mass spectrometric analysis of complex tissue samples, is the difficulty to identify the protein for which molecular mass is detected.

B. Enrichment in Sub-Cellular Structures

Sub-cellular fractionation strategies include a variety of established and innovative approaches that are made particularly effective in combination with Bio-MS to profile protein constituents. With traditional approaches, generally the sample is first subjected to homogenization to obtain a free suspension of intact, individual organelles by means of low-speed centrifugation. The nuclei, together with cell debris and unbroken cells, are removed as a pellet. The supernatant that contains the cytosol and other organelles in suspension is subjected to sub-cellular fractionation. Density-gradient centrifugation is the most popular approach to efficiently perform sub-cellular fractionation. The sub-cellular fraction(s) of interest is enriched, and is subjected to further separation of constituent proteins, coupled with Bio-MS.

1. Density-Gradient Centrifugation for Sub-Cellular Fractionation

Fractionation by gradient centrifugation, based on the sedimentation velocity of organelles in gradient medium such as sucrose and percoll, has been frequently applied to the fractionation of sub-cellular organelles, such as Golgi and mitochondria. Bergeron's group has utilized sucrose density-gradient centrifugation to isolate different organelles. In studies of the Golgi organelle, membrane proteins were resolved by 1D SDS

electrophoresis. Bands of interest were analyzed by MALDI-TOF MS or Q-TOF MS/MS. A total of 81 membrane proteins, including a novel Golgi-associated protein of 34 kDa (GPP34), were unambiguously identified (Dominguez et al., 1999; Bell et al., 2001). An abundance of trafficking proteins was uncovered, such as KDEL receptors, p24 family members, SNAREs, Rabs, ARF-guanine nucleotide exchange factor, and SCAMPs. Hanson and Lescuyer used sucrose density-gradient centrifugation to analyze mitochondrial proteins in combination with 2D PAGE and Bio-MS. Functional information on protein complexes within human brain mitochondria was obtained (Hanson et al., 2001). The human mitochondrial proteome map, using placenta as the source tissue, was recently constructed and a large number of proteins were identified, including novel ones (Lescuyer et al., 2003). Andersen and his colleagues reported their direct study of the human sub-nuclear proteome by using a combination of sonication and sucrose density-gradient centrifugation to fractionate nucleoli from HeLa cell nuclei, followed by 1D or 2D gel protein separation and Bio-MS analysis (Andersen et al., 2002). A total of 271 proteins were identified, and more than 30% of the nucleolar proteins were encoded by novel or uncharacterized human genes.

Murayama et al. (2001) described a novel approach that uses freeze-thawing to produce a density-gradient solution of Nycodenz for the one-step fractionation of organelles from rat liver and subsequent analysis of fractions by 2D PAGE. An alternative technique that used differential centrifugation and hypotonic lysis was applied to separate lysosomes from endosomes and pre-lysosomal compartments (Schafer & Heizmann, 1996). This approach resulted in a pure lysosomal fraction that contained high specific activities of lysosomal enzymes, and an endosomal fraction that contained endosomes at different stages without detrimental effects on the quality of the isolated fractions. This sub-cellular pre-fractionation technique is applicable to a variety of human cell populations.

2. Immune-Based Sub-Cellular Fractionation

Immune-based techniques use the high specificity of antibodies to capture sub-cellular organelles that contained the cognate antigen. Shevchenko et al. (1997) used this approach to isolate trans-Golgi network (TGN)-derived apical and basolateral transport vesicles, followed by 2D PAGE and Bio-MS. Two proteins that belong to the p23/p24 family of putative cargo receptors for vesicular trafficking were identified, and caveolin-2 was also characterized as a constituent of basolateral transport vesicles. This approach can also be used in combination with gradient centrifugation to further reduce protein cross-contamination from different organelles, such as endoplasmic reticulum, Golgi membrane, and plasma-membrane proteins.

3. Free-Flow Electrophoresis for Sub-Cellular Fractionation

Free-flow electrophoresis (FFE) is a revitalized old technique that is based on differences in electrophoretic mobility between various components in mixtures, ranging from polypeptides and sub-cellular organelles to cells. Thus, a variety of sub-cellular organelles may be separated on the basis of their unique charge density. For example, lysosomes of human skin fibroblasts were efficiently isolated by FFE after differential centrifugation of the cell lysate suspended in isotonic sucrose (Harms, Kern, & Schneider, 1980). Marsh et al. (1987) described a rapid sub-cellular pre-fractionation approach that combined density-gradient centrifugation with FFE to isolate endosomes from a variety of tissue culture cells. The post-nuclear supernatants were subjected to FFE. Endosomes and lysosomes migrated together as a single anodally deflected peak separated from most other organelles such as plasma membrane, mitochondria, endoplasmic reticulum, and Golgi. The endosomes were further resolved from lysosomes by centrifugation in a percoll density gradient. A 70-fold enrichment of endosomes was achieved relative to the initial homogenate. FFE was also used to separate flagellar pocket-derived membranes from other endosomal and lysosomal organelles of African trypanosomes (Grab, Webster, & Lonsdale-Eccles, 1998).

Immune free-flow electrophoresis (IFFE) combines the advantages of electrophoretic separation with the high selectivity of antigen–antibody binding. It relies on the altered electrophoretic mobility of a sub-cellular organelle complexed to a specific antibody against the cytoplasmic domain of one of its integral membrane proteins when the electrophoresis buffer pH is adjusted to 8.0, close to the p*I* of immunoglobulin (Ig). Thus, Ig-coupled organelles can be separated from other structures by FFE. Mohr and Volkl applied IFFE to the isolation and analysis of peroxisomes as well as of microsomal fractions obtained by differential centrifugation of a rat liver homogenate (Volkl, Mohr, & Fahimi, 1999; Mohr & Volkl, 2002).

Another variation on the theme, density-gradient electrophoresis (DGE), combines the principle of FFE with density gradients (Tulp, Verwoerd, & Pieters, 1993). After homogenization, organelle mixtures are layered within a sucrose or Ficoll gradient that is subjected to electrophoresis. Endosomal and lysosomal organelles, being negatively charged, migrate preferentially towards the anode and are separated from other sub-cellular organelles. Tulp et al. (1998) applied high-resolution DGE for the sub-cellular fractionation of late endosomes, early endosomes, lysosomes, endoplasmic reticulum, plasma membrane, clathrin-coated pits, proteasomes, and clathrin-coated vesicles from the postnuclear supernatant, by using a novel low-conductivity buffer. A DGE protocol was developed that allowed a one-step separation of plasma membrane, Golgi/TGN (Lindner, 2001) and endosomes for the quantitative analysis of vesicular transport from the Golgi/TGN compartment to the plasma membrane and endosomes (Lindner, 2001).

IV. FRACTIONATION OF PROTEINS BASED ON THEIR CHEMICAL AND PHYSICAL PROPERTIES REDUCES COMPLEXITY AND ENHANCES THE YIELD OF LOW-ABUNDANCE PROTEINS

The protein complexity of biological samples may be simplified through fractionation based on different protein properties, including sequential solubilization, selective precipitation, and affinity purification, or through various chromatography-based

methods (Issaq et al., 2002; Liu et al., 2002; Wang & Hanash, 2003).

A. Chromatography-Based Fractionations

Virtually all chromatographic modalities have been used for the pre-fractionation of biological samples in order to achieve an enhanced resolution of proteins in individual fractions. RP-HPLC has been applied to fractionate protein mixtures of tissue lysates, and each fraction was presented to 2D PAGE for further separation (Badock et al., 2001; Van Den Bergh et al., 2003). Some low-abundance proteins were enriched in 2D gels and were identified by Bio-MS (Badock et al., 2001). In another study, RP-HPLC pre-fractionation was applied to visual cortex tissue lysates prior to analysis. Some protein spots that were not observed in total tissue lysates were visualized and identified (Van Den Bergh et al., 2003).

SEC has also been used as a pre-fractionation technique. For example, the human lens proteins crystallins become extensively modified with aging, and the characterization of these modified proteins is of significance because they are the likely precursors of cataract. In one study, the soluble crystallins were first fractionated into α-, β-, and γ-crystalins by SEC (Zhang, Smith, & Smith, 2001). All of the β-crystallins, including three acidic subunits (βA1, βA3, βA4) and three basic subunits (βB1, βB2, βB3), were collected into one fraction, and were further fractionated by RP-HPLC. The βA4 and βB1 RP-HPLC fractions were separated by 2D PAGE, followed by the characterization of the spots of interest. IEC separates protein mixtures based on charge in a non-denaturing environment. Proteins with similar p*I* and strongly associated proteins are co-eluted in the same fraction. In one study, IEC pre-fractionation simplified the complexity of whole cell lysates for the analysis of multi-protein complexes by 2D PAGE, and also resulted in protein enrichment for subsequent mass spectrometric analysis (Butt et al., 2001).

Chromatofocusing (CF) is a type of IEC that separates proteins according to their p*I*. Proteins bound to the gel matrix are eluted with a specific poly-buffer in the order of decreasing p*I*. Fountoulakis et al. used CF to fractionate and enrich *Haemophilus influenzae* protein mixtures. Proteins were further separated by 2D PAGE. Seventy new proteins were identified in the CF pools, many of which occurred in low abundance and were not detectable by the direct analysis of lysates by 2D PAGE (Fountoulakis et al., 1998). Similarly, that same group used hydrophobic interaction chromatography (HIC) to separate *Haemophilus influenzae* proteins based on their hydrophobicity, followed by 2D PAGE and MALDI-TOF MS to identify novel proteins (Fountoulakis, Takács, & Takács, 1999).

B. Liquid-Phase Electrophoresis Prior to SDS–PAGE

FFE has been used to fractionate protein mixtures based on p*I*. Hoffmann and his colleagues used continuous FFE to isolate cytosolic proteins from the human colon carcinoma cell line LIM 1215 into 96 fractions, followed by SDS–PAGE gel separation. The resolved proteins were identified by peptide fragment sequencing, using on-line capillary LC-MSMS (Hoffmann et al., 2001). The experimental relative molecular mass (*M*r) and p*I* of identified proteins were in good agreement with the theoretical values calculated from the amino acid sequence.

Bae et al. applied the Gradiflow technique, another liquid-phase IEF separation applicable to proteins with p$I > 9.0$ (Locke et al., 2002) to fractionate proteins from *H. pylori* whole cell lysates to obtain two groups of basic proteins followed by further separation with SDS–PAGE. Sixteen bands were selected for protein characterization by MALDI-TOF MS (Bae et al., 2003). Seventeen basic proteins were identified, five of which (HP1216, HP1283, Cag3, Cag13, and KataA) with predicted p*I*s between 8.97 and 9.69 were not identified on either pH 6–11 or pH 9–12 IPG 2D PAGE gels without sample pre-fractionation.

C. Liquid-Phase IEF Prior to Hybrid Multi-Dimensional Separation

An orthogonal high-resolution multi-dimensional separation system developed in our laboratory, using liquid-phase IEF as the pre-fractionation approach followed by separation with RP-HPLC and SDS–PAGE, was applied to cancer cell-line lysates or bio-fluids. This system was coupled with protein biochips and Bio-MS to probe the human cancer proteome, as illustrated in Figure 2 (Madoz-Gurpide et al., 2001). The advantage of the combination of liquid-phase IEF with RP-HPLC is that proteins in hundreds of individual fractions can be arrayed directly and used as targets for a variety of probes. Constituent proteins in reactive fractions of interest are subjected to further separation on SDS gels followed by protein characterization of bands of interest by capillary LC ESI Q-TOF MS/MS (Nam et al., 2003). We have also used this separation system in combination with fluorescence detection to quantitatively profile the human serum proteome (Wang et al., 2002). Two independent serum samples, such as a study sample and a control, are labeled with different fluorescence reagents such as Cy3 and Cy5, and combined prior to three-dimensional protein separation. The resolved proteins are visualized in SDS gels, and protein bands of interest are analyzed by either MALDI-TOF MS or capillary LC ESI Q-TOF MS/MS (Fig. 3).

D. Affinity and Immunocapture

Affinity- and immunocapture-based methods represent a well-established approach to enrich protein subsets of interest. With these approaches, complexity may be reduced to such a large extent that a simple one-dimensional separation procedure may be sufficient to resolve captured protein constituents. For example, Pandey et al. (2000) used immunoprecipitation techniques to precipitate tyrosine-phosphorylated proteins and to probe signaling-related cell-surface receptors by 1D SDS gel/Bio-MS. Journet used mannose-6-phosphate (M-6-P) receptors (MPRs) as the affinity chromatography separation media to specifically purify M-6-P proteins from soluble human U937 cell lysosomal hydrolase. The captured proteins were separated by 2D PAGE, and were analyzed with either MALDI-TOF MS or ESI Q-TOF MS/MS. Twenty-two proteins were identified, among which 16 were well-known lysosomal hydrolases, such as proteinase 3, cathepsin A, cathepsin D, cathepsin S, Dnase II, β-glucuronidase, and acid ceramidase (Journet et al., 2002). Phosphospecific

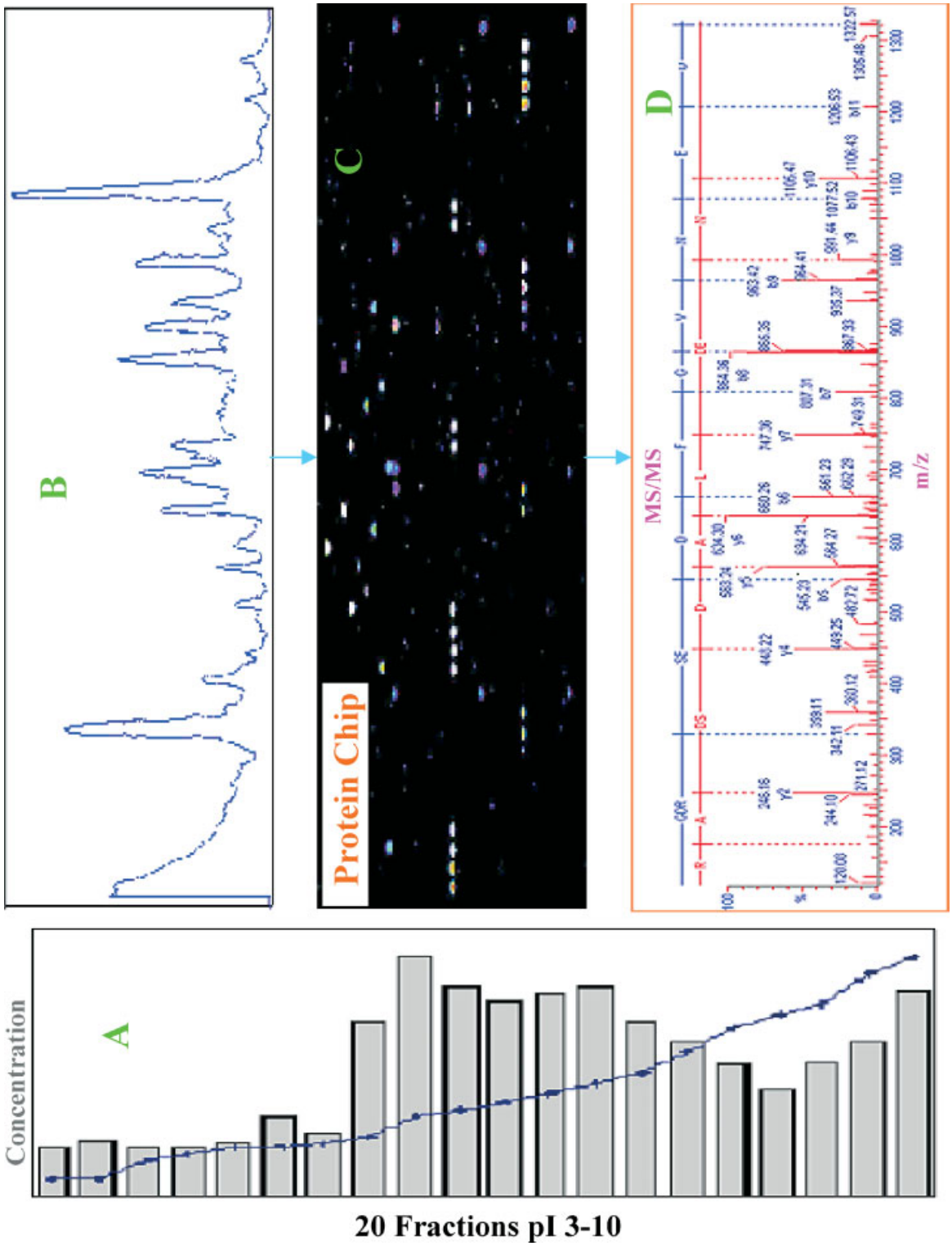

FIGURE 2. Multi-dimensional separation system coupled with protein biochip and Bio-MS. **A**: Cell or/and tissue lysates are separated into 20 fractions by iso-electric focusing; (**B**) individual fractions are further resolved by reversed-phase HPLC; (**C**) aliquots of separated proteins are arrayed onto glass slides for subsequent probing, uncovering in this case spotted proteins that react with antibodies in a cancer subject's serum. **D**: Individual fractions of interest are further analyzed by mass spectrometry, showing in this case a mass spectrum for a peptide digest from a spotted protein.

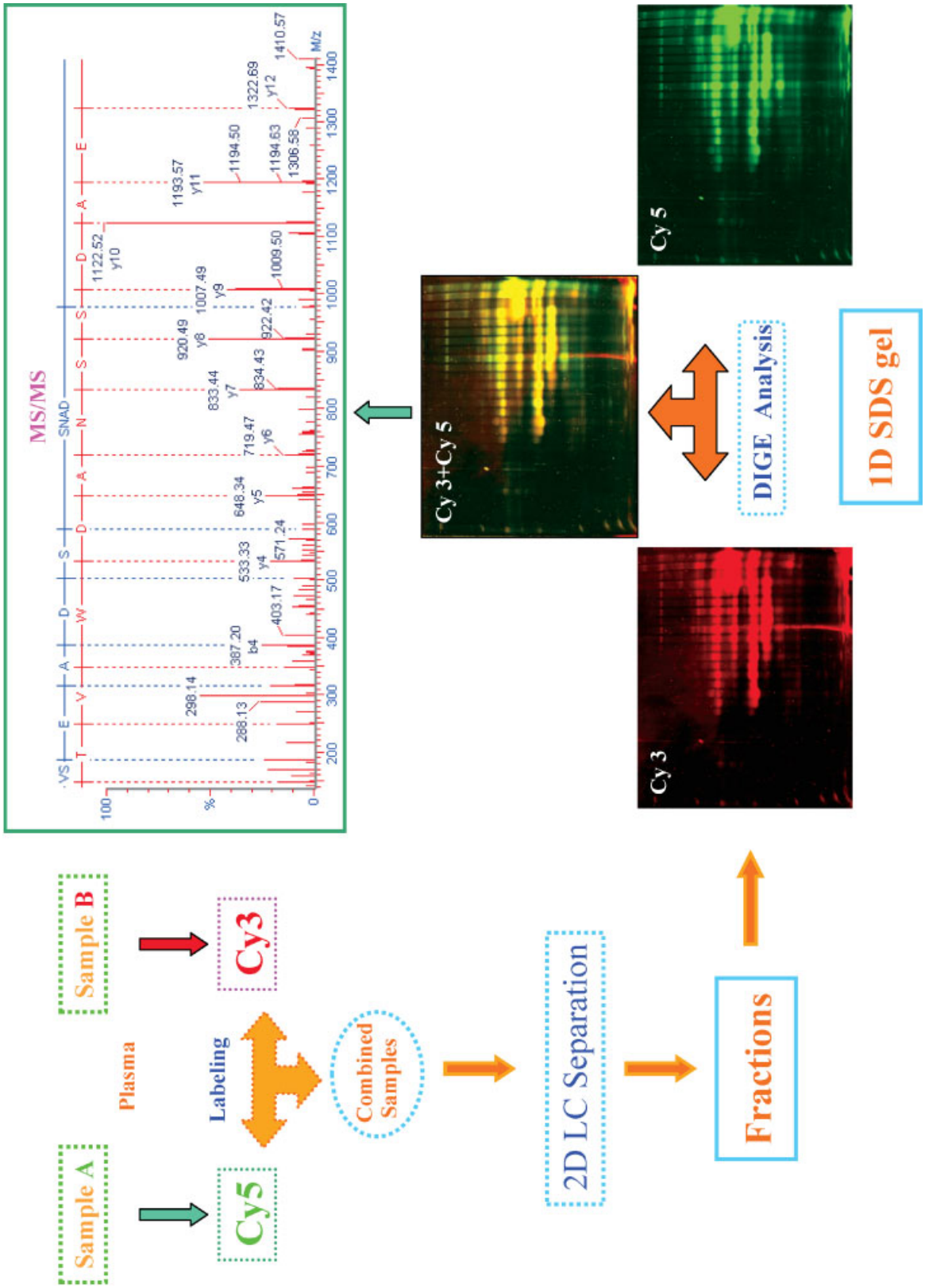

FIGURE 3. Multi-dimensional separation system combined with 2D differential in-gel electrophoresis (DIGE)/Bio-MS for human plasma proteome analysis.

antibody-based immunoaffinity chromatography and immobilized metal-based affinity chromatography (IMAC) have been used to enrich phosphoproteins and to decipher the phosphoproteome with Bio-MS-based strategies (Kalume, Molina, & Pandey, 2003). Heparin affinity chromatography was used to enrich low-abundance human fetal brain protein mixtures, and the eluted affinity-specific fractions were resolved by 2D PAGE. Approximately 70 enriched unique proteins that belong to several classes, including proteasome components, dihydropirimidinase-related proteins, and T-complex protein I components, and enzymes with various catalytic activities were identified by MALDI-TOF MS (Karlsson et al., 1999). Shefcheck, Yao, & Fenselau (2003) used heparin affinity chromatography to fractionate cytosolic protein mixtures of human MCF-7 breast cancer cells into three fractions prior to 2D PAGE. A striking level of enrichment is achieved for low-abundance proteins in each fraction, and 300 proteins were visible in 2D gel patterns of the three fractions. Those 300 proteins could not be detected in non-fractionated cytosol.

E. Depletion/Enrichment of Proteins From Human Plasma/Serum

There is an increasing interest focused on the human plasma/serum proteome because of the great relevance of plasma/serum proteins to biomedicine, including diagnostics and therapeutic monitoring (Adkins et al., 2002). However, high-abundance proteins in plasma/serum, including albumin, immunoglobulins (IgG and IgA), antitrypsin, transferring, and haptoglobin, interfere with the proteomic analysis of low-abundance proteins because of the consequent loss of resolution in 2D PAGE or chromatographic separations. Thus, most of the interesting low-abundance proteins ($\leq$1 ng/mL level) are not detected. Therefore, it is advantageous to specifically remove high-abundance proteins in a sample pre-fractionation step prior to protein analysis. Specific removal of high-abundance proteins will deplete approximately 85–90% of the total protein mass from human plasma/serum. Pieper et al. (2003) reported an elegant quantitative strategy to selectively profile low-abundance proteins in human plasma by a multi-component immunoaffinity chromatography approach, based on antibody–antigen interactions, to deplete 10 high-abundance proteins from plasma. Affinity-purified polyclonal antibodies (pAbs) were used as the stationary phase in the column to specifically capture high-abundance proteins albumin, IgG, IgA, transferrin, α-1-antitrypsin, haptoglobin, α-2-macroglobulin, hemopexin, α-1-acid glycoprotein, and α-2-HS glycoprotein. This specific step of selective immunodepletion provided an enriched pool of low-abundance proteins in the flow-through fraction from the column for subsequent 2D PAGE/Bio-MS analysis. An increment of 350 low-abundance proteins was visualized after depletion.

Wang & Hanash (2003) developed a simple and rapid immunoaffinity-based method, using an affinity spin-tube filter to deplete high-abundance proteins or to enrich low-abundance protein biomarkers in human serum. The affinity spin-tube filter contains protein G, coupled with antibodies against either high-abundance proteins for the depletion of proteins like albumin and IgG, or specific proteins of interest for the enrichment of low-abundance protein biomarkers such as fatty acid synthase (FAS). The flow-through fraction or the eluate was recovered and subjected to 2D PAGE for further separation. Of total albumin 88% was depleted from the serum with enhanced detection of the remaining proteins. Total protein recovery was >95%.

Rothemund et al. (2003) used the Gradiflow technique to deplete albumin from human plasma on the basis of its p*I* and *M*r. Human plasma proteins were fractionated into four groups: albumin, proteins with p*I* greater than albumin, proteins with *M*r higher than albumin, and proteins with *M*r lower than albumin. The albumin-depleted fractions were separated by 2D PAGE to allow the detection of low-abundance proteins. A chain of protein spots that lay beneath albumin in non-fractionated plasma were revealed, and allowed the identification of C4B-binding protein α chain. One advantage of using Gradiflow for the depletion of albumin is its ability to separate proteins by *M*r to allow some proteins to be separated from albumin even though their p*I*s were close to that of albumin. The Human Proteome Organization (http://WWW.HUPO.org) is currently assessing the merits of various depletion protocols as part of the Human Plasma Proteome Project.

V. FRACTIONATION OF PROTEINS BASED ON THEIR POST-TRANSLATIONAL MODIFICATIONS

PTMs, such as glycosylation, phosphorylation, sulfation, and acetylation, modulate important biological activity of proteins during cellular processes. Identification of sites of protein PTMs and the quantitative analysis of modified proteins would provide insight into biological functions. There are currently two major affinity chromatography-based techniques coupled with Bio-MS for the analysis of proteins. One is the IMAC and the other is the isotope-coded affinity tagging (ICAT) technique (Fiacre et al., 2002; Kalume, Molina, & Pandey, 2003; Mann & Jensen, 2003). These approaches are outside the scope of this review. However, from the biomedical applications point of view, there is still a substantial need to develop highly efficient approaches for the quantitative analysis of PTMs.

VI. PROTEIN-TAGGING STRATEGIES

Protein-tagging techniques have great utility to capture protein subsets and/or to enhance sensitivity. They have been applied to the study of multi-protein complexes (Bauer & Kuster, 2003), comprehensive profiling of subcellular proteomes (Shin et al., 2003), and for quantitative profiling of overall protein expression (Unlu, Morgan, & Minden, 1997).

A. Biotin Tags to Profile Cell-Surface Proteins

Cell-surface proteins are involved in a multitude of intercellular and extracellular functions. However, the global proteomic analysis of this compartment has been quite challenging because of the intrinsic features of its protein constituents, including high hydrophobicity and low expression levels. Thus, the development of efficient sample-preparation techniques to isolate intact

surface membrane proteins as well as other membrane proteins is of substantial interest. Our group has implemented an intact protein-based strategy that incorporates the capture of cell-surface proteins by biotinylation followed by biotin–avidin affinity chromatography and the visualization of captured proteins by 2D PAGE or other protein separation strategies as outlined in this review. This strategy has been successfully applied to the global profiling of the cell-surface proteome of cancer cells that belong to different lineages (Shin et al., 2003). Figure 4 shows the strategic scheme to profile the cell-surface proteome, using affinity chromatography-2D PAGE-capillary LC-MS/MS. The surface proteins of viable, intact cells are subjected to biotinylation with EZ-Link sulfo-NHS-LC-biotin *in vitro* at 37°C for 10 min, followed by the affinity capture of solubilized biotinylated surface-membrane proteins with a monomeric avidin column. The enriched biotinylated proteins may be visualized in 2D gels as intact proteins to allow the elucidation of their differential expression and assessment of their PTMs. Alternatively, they may be fractionated with a liquid-based strategy. Interestingly, a set of chaperone proteins previously associated with the endoplasmic reticulum, including GRP94, GRP78, GRP75, HSP90A/B, HSP70, HSP60, HSP54, HSP27, and protein disulfide isomerase A3/A6, were found to be abundantly expressed on the surface of cancer cells. Other novel proteins with a more restricted expression were also identified. Sabarth et al. (2002) have applied a similar biotinylation approach to the identification of surface-membrane proteins recovered from *Helicobacter pylori*. Eighty-two biotinylated proteins were resolved by 2D PAGE, and a total of 18 proteins was characterized by MALDI-TOF MS.

B. Fluorescent Tags for Comparative Proteomics

Conventional methods for the comparison of 2D gel images from different samples traditionally have involved the analysis of one sample per gel. Because of variability between gels, the detection and quantification of protein differences can be problematic. Unlu and his colleagues developed a differential in-gel electrophoresis (DIGE) technique that involved: (1) fluorescently

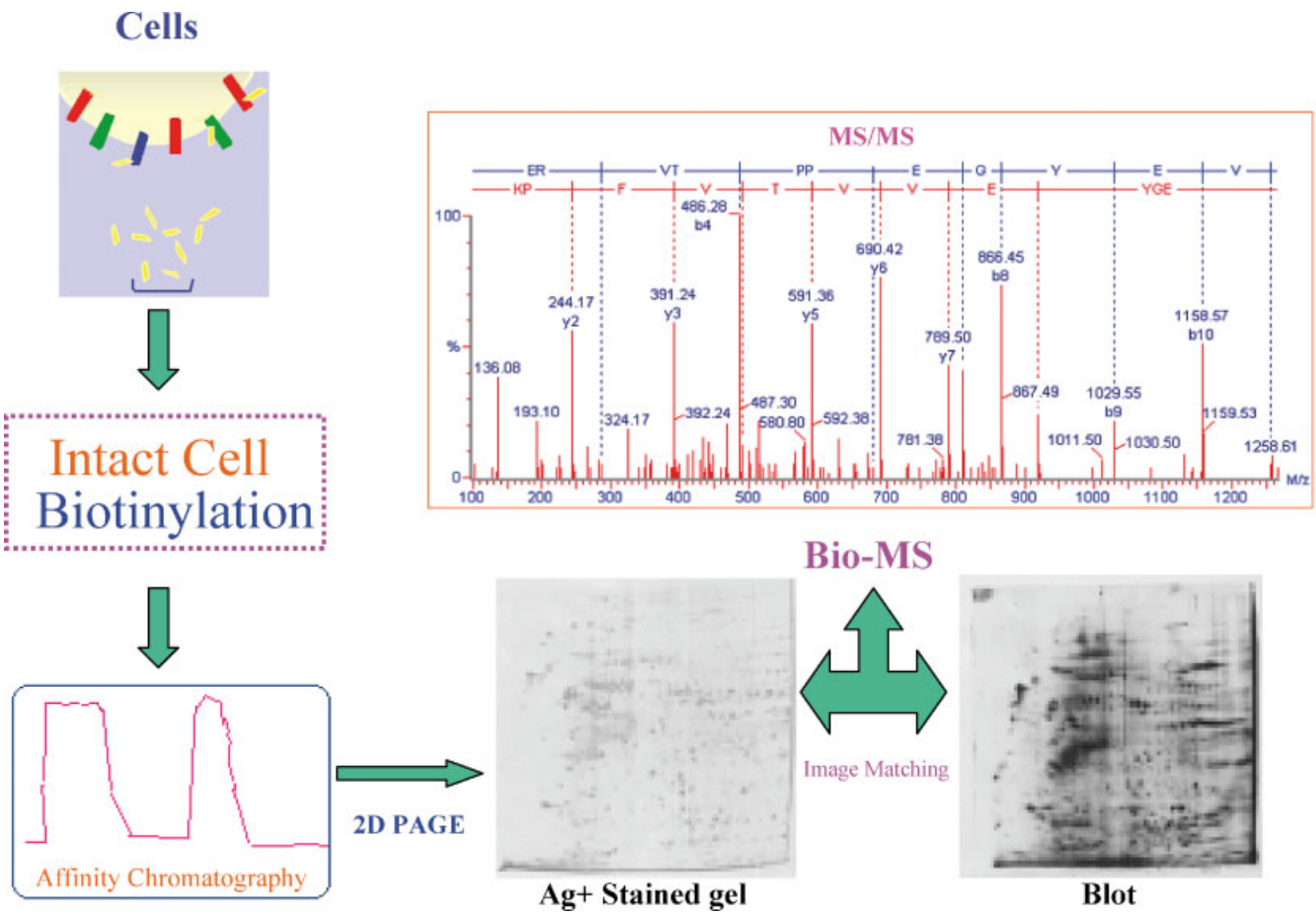

FIGURE 4. Affinity chromatography prior to two dimensional poly-acrylamide gel electrophoresis (2D PAGE)/Bio-MS to profile the cell-surface proteome. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

tagging two protein samples with two different fluorescent dyes (such as Cy3 and Cy5), (2) separating them on the same 2D gel (2D DIGE), (3) fluorescence imaging of the gel into two images, and superimposing the images to detect any differential expression between the two protein samples (Unlu, Morgan, & Minden, 1997). This method improves the reproducibility and reliability of differential protein expression analysis between samples, and is particularly useful in comparative studies of normal and diseased tissues. Several groups have used the 2D DIGE technique in various studies, such as analysis of mouse-liver homogenates treated with *N*-acetyl-*p*-aminophenol (APAP) (Tonge et al., 2001), elucidation of the effect of ErbB-2 over-expression on breast cancer cells (Gharbi et al., 2002), and analysis of bacteria under different growth conditions (Gade et al., 2003). Yan et al. (2002) used this technique to study the *Escherichia coli* proteome after benzoic acid treatment. A total of 179 differentially expressed protein spots were identified. Those proteins included enzymes, stress-related, and substrate-binding proteins (e.g., amino acids, maltose, ribose, and TRP repressor).

VII. PERSPECTIVE AND CONCLUSION

It has become obvious exceedingly that there are no simple universal strategies for the comprehensive analysis of complex proteomes. There are specialized strategies—each with some advantages and some disadvantages. The merits of these strategies must be weighed in relation to the contemplated specific applications. In this review, we have presented intact protein-based strategies that are intended to reduce the complexity of biological samples prior to MS. Such strategies have substantial versatility because they allow the detection, identification, and recovery of proteins of interest. It should be pointed out that a relatively small subset of proteins, particularly large hydrophobic membrane proteins, may defy analysis with such a strategy, and their investigation may be better-suited to other approaches. However, these proteins likely represent a small proportion of the proteome of complex organisms. For most proteins, and in particular for biological fluids, it may be possible in the future to eliminate the need for digestion altogether, for example, by interfacing intact protein-separation strategies directly with MS, where proteins are first assessed with respect to their intact mass followed by their fragmentation that process is to derive their identity as well their PTMs. Additionally, microfluidics and nanotechnologies have yet to make an impact on proteomics, despite their substantial appeal. Nevertheless, it may also be envisioned that such technologies will make, in the not so distant future, as much of an impact on proteomics as MS has and we look forward to substantial miniaturization and automation of intact protein-based strategies for the analysis of complex proteomes.

VIII. ABBREVIATION LIST

1D	one dimension
2D	two dimension
2D PAGE	two dimensional poly-acrylamide gel electrophoresis
Bio-MS	biological mass spectrometry
CE	capillary electrophoresis
CF	chromatofocusing
CID	collision-induced dissociation
CSMPs	cell-surface membrane proteins
Cy3	1-(5-carboxypentyl)-1′-propylindocarbocyanine halide *N*-hydroxysuccinimidyl ester
Cy5	1-(5-carboxypentyl)-1′-methylindodicarbocyanine halide *N*-hydroxysuccinimidyl ester
CSF	cerebrospinal fluid
DGE	density-gradient electrophoresis
DIGE	differential in-gel electrophoresis
ESI	electro-spray ionization
FAB	fast atom bombardment
FFE	free-flow electrophoresis
FTICR	Fourier-transform ion cyclotron resonance
GRP78	78 kDa glucose-regulated protein
HIC	hydrophobic interaction chromatography
HSP90B	heat-shock protein HSP 90-beta
HUPO	Human Proteome Organization
ICAT	isotope-coded affinity tag
IEC	ion-exchange chromatography
IEF	isoelectric focusing
IFFE	immune free-flow electrophoresis
IMAC	immobilized metal affinity chromatography
LC	liquid chromatography
LCM	laser-capture microdissection
LC-MSMS	liquid-chromatography combined with tandem mass spectrometry
M-6-P	mannose-6-phosphate
MALDI-TOF	matrix-assisted laser desorption/ionization-time-of-flight
MCE	multi-compartment electrolyzer
MS	mass spectrometry
MSMS	tandem mass spectrometry
PTMs	post-translational modifications
Q-TOF	Quadrupole-time-of-flight
RPLC	reversed-phase liquid chromatography
SEC	size-exclusion chromatography

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