An introduction into proteomics and its clinical applications

Mamoun Ahram, PhD.

ABSTRACT

The completion of the human genome project has marked the official start of the post-genomic era. Due to limitations in DNA and RNA studies, the advent of proteomics, or large-scale analysis of proteins, is considered a crucial consequence and a chief player of post-genomic initiatives. An immediate goal of proteomic studies is the understanding of proteins including their expression, function, interaction, and structure with an endpoint of discovery of protein biomarkers. Such biomarkers can be used in detection, prognostication, and treatment of diseases. However, the challenge of studying complete sets of cellular proteins, or proteomes, is driving the development of newer technologies. In this review, a discussion of proteomic biotechnologies and their clinical applications is offered. The main techniques covered are the 2-dimensional polyacrylamide gel electrophoresis, mass spectrometry, surface-enhanced laser desorption ionization mass spectrometry, and protein microarrays. These tools have been successfully utilized in analyzing human and other biological samples in discovery of biomarkers. Recent advancement in proteomics has added and will continue to add valuable information to our knowledge-base of the human biological system.

Saudi Med J 2007; Vol. 28 (4): 499-507

From the Department of Pharmacology and Physiology, Faculty of Medicine, Mutah University, Mutah, Karak, Jordan.

Received 14th August 2006. Accepted 16th December 2006.

Address correspondence and reprint request to: Dr. Mamoun Ahram, Department of Pharmacology and Physiology, Faculty of Medicine, Mutah University, Mutah, PO Box 7, Karak 61710, Jordan. Tel. +962 (79) 5567779. Fax. +962 (3) 2386175. E-mail: Dr.ahram@gmail.com

The completion of the human genome project has generated tremendous excitement in the scientific community with the many opportunities it provides not only in unraveling the human genome, but also in understanding the complexity of the human biological system as a whole. However, many deficiencies exist in regards to the basic knowledge of human genes. First of all, of the 20,000-30,000 genes that make up the human genome, the expression pattern in different cells and tissues has yet to be determined. It has been estimated that ~6,000 genes are expressed per cell with a cell specificity of ~400 proteins.[1] This estimate is reached without considering the protein end-products following post-translational modifications. In addition to lack of knowledge in regards to gene expression, the function of many of the human genes has not been elucidated. A closer examination of a human protein database reveals the presence of a considerable fraction of hypothetical gene products, which has been estimated to be approximately 60% of a database.[2]

Major modern scientific research efforts are aimed towards "molecular profiling", or global measurements of mRNA and proteins levels in biological systems.[3,4] This strategy is based on information provided by the human genome project and aided by development of technologies for molecular analysis of diseases. Current technologies used in molecular profiling studies generate enormous amount of data points in a short period of time, thus termed "high-throughput." High-throughput studies allow the concurrent investigation of thousands of genes enhancing the discovery of novel genes and elucidation of molecular and cellular interactions. However, due to the relative biochemical consistency of RNA molecules and the ease of mRNA manipulation in contrast to proteins, molecular profiling studies and technological developments have mainly been driven towards determination of mRNA levels.

Although RNA-based studies are promising in identifying gene products linked to diseases, such studies suffer from serious shortcomings. First, it is important to realize that mRNA molecules are transit products into making proteins, the primary functional player in cells. In addition, RNA transcript levels may not correspond to real protein levels due to differences in regulation and stability. This has been illustrated in previous studies where levels of mRNA molecules and their corresponding proteins poorly correlate.[5,6] In addition, proteins may undergo extensive modification such as proteolysis, glycosylation,

or phosphorylation generating different isoforms each with distinct function. The same protein may also differ in its localization in physiological and pathological conditions influencing its function. Finally, proteins are the main target of therapeutic agents accounting for more than 98% of drug targets.[7] Due to the biological significance of proteins, the field of "proteomics" has launched.

Many attempts have been made to define the term, "proteomics." A simple definition can be stated as "high-throughput analysis of proteins" involving hundreds to thousands of proteins. Among the many goals of proteomics is understanding of all aspects of proteins including their expression, function, molecular interaction, and structure. It is hoped with proteomic analyses to discover novel disease biomarkers that can be utilized for detection, prognostication, and treatment of various diseases. The challenge of studying complete sets of proteins in cells, or proteomes, has been driving the development of new technologies. In fact, proteomics can be considered an application of evolving biotechnologies. Continuous improvement of older instruments has facilitated the execution of prominent proteomic studies. In addition, newer and powerful instruments have also been developed. In this review, commonly used proteomic technologies and examples of their applications are introduced. The clinical aspect of these techniques will also be emphasized. As will be noted, no proteomic technique will reveal the mystery of our proteomes and, therefore, multiple methods are needed to achieve an appreciable level of knowledge.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Two-dimensional polyacrylamide gel electrophoresis is rather an older protein analytical tool invented more than 30 years ago.[8,9] However, since mid 1990's, it has been revived and been the proteomic tool of choice in many laboratories (for review).[10] This technique enables the separation of proteins in 2-dimensions based on their isoelectric point (first dimension) followed by their mass (second dimension) (**Figure 1**). A few hundreds to approximately two thousands of proteins can be visualized by 2D-PAGE producing a "protein fingerprint" of cells. Major improvements have been introduced making 2D-PAGE technically user-friendly and reproducible. For example, the development of immobilized pH gradient (IPG) strips has allowed consistent and reproducible focusing of proteins in the first dimension.[11] In addition, protein focusing can be performed at different ranges of isoelectric points such as a standard 3-10 range or narrower ranges including 4-7, 5-8, and 6-11, allowing wider separation and, hence, the visualization more proteins.

Once separated in the second dimension by a standard polyacrylamide-based gel electrophoresis, proteins can then be visualized using a staining dye. Currently, there are 3 types of staining procedures commonly used for detection of protein spots. The first is based on a blue dye known as Coomassie blue. This relatively inexpensive procedure is simple and allows the detection of proteins as low as 1 ng. The second staining method is based on silver nitrate requiring more elaborate work than the former method, but is more sensitive where proteins as low as 0.1 ng can be detected. However, due to the utilization of a fixative in the silver staining method, the sensitivity of protein identification by mass spectrometry (to be covered later) is significantly compromised. As a result, a fluorescent method using a dye known as Sypro has been reported (Molecular Probes, Eugene, Oregon, USA, http://probes.invitrogen.com/) providing the same sensitivity of silver staining and the simplicity of Coomassie blue staining.[12,13] A drawback of fluorescent dyes, however, is their high expense relative to the other 2 methods.

It is now possible to analyze 2 samples, namely, normal and diseased, by the same 2D gel with the commercial introduction of 2-dimensional differential in-gel electrophoresis (DIGE).[14,15] This technique overcomes problems associated with comparative quantification of protein spots in two different gels due to preparative and staining artifacts. In DIGE, the proteins of 2 samples are labeled with different fluorescent tags. Then, they are combined at equal amounts and separated by 2D-PAGE as is normally performed for a single-sample experiment. The gel can be visualized under a special imaging system where each sample is scanned independently and the images are superimposed for comparative, computational analyses. DIGE has been utilized in discovery of protein biomarkers in breast and esophageal cancers.[16,17]

Celis et al,[18] have illustrated the occurrence of major modifications in protein expression patterns when cells are removed from their natural in vivo environment. Similar observations are made when the protein profile of prostate cells isolated from a tissue sample is compared to that of the same cells but grown in vitro.[19] Thus, direct proteomic studies of human tissues are critical, in particular, since tissues are the true representation of physiological and pathological states. The advent of Laser Capture Microdissection (LCM; Arcturus Engineering, http://www.arctur.com/) has made it possible to study protein profiles of pure populations of cells procured from tissue sections (**Figure 2**).[20] Laser Capture Microdissection has been successfully utilized in discovery of protein biomarkers including those

of prostate and esophageal cancers.[21,22] The source of tissues has been shown to not only be limited to frozen samples, but can also include ethanol-fixed, paraffin-embedded tissues.[22,23]

Although 2D-PAGE is an extremely powerful technique, it suffers from major shortcomings. These include the inability to resolve hydrophobic as well as highly basic proteins. In addition, 2D-PAGE can detect a small fraction of a cellular proteome. For example, if a cell consists of 6,000 proteins as hypothesized,[1] 2D-PAGE can detect no more than one-third of the proteome in perfect circumstances. This is a significant limitation if we consider that intermediate and low-abundance proteins such as protein kinases and growth factors are major players in human diseases. 2D-PAGE is also limited to analyzing proteins larger than 5 kDa.

One dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D-SDS-PAGE). Due to limitations in analysis of certain protein classes by 2D-PAGE, the traditional 1D electrophoretic method, namely SDS-PAGE, has been employed in a number of proteomic studies. Sodium dodecyl sulfate is an ionic detergent that has proven superior in general protein solubilization. In this technique, proteins are resolved based on mass only. For large-scale proteomic studies, the gel lane containing separated proteins is cut into smaller pieces and proteins in each piece are identified by mass spectrometry. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis has led to the identification of numerous proteins from membrane preparations of colon cancer cells[24] and breast cancer.[25] For review on proteomic analysis of membrane proteins, refer to Ahram and Springer.[2] However, similar to 2D-PAGE, SDS-PAGE is limited to analyzing proteins larger than 5 kDa. In addition, due to resolving and staining limitations of SDS-PAGE, it is not practical to perform visual comparison of individual proteins, especially for crude samples.

Mass spectrometry (MS). Currently, MS is currently the method of choice in high-throughput proteomic studies. A mass spectrometer measures the masses of small molecules by converting them into ions and sorting

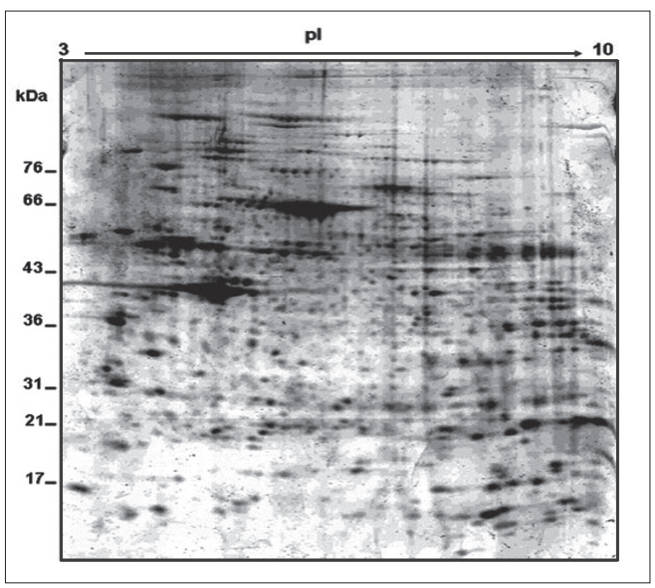

Figure 1 - Protein analysis by 2-dimensional polyacrylamide gel electrophoresis. Proteins are separated by in 2 dimensions based on 2 biochemical features: isoelectric point or charge (first dimension) and mass (second dimension). In the illustrated gel, proteins are separated at a range of pH 3-10 in the first dimension and 5-100 kDa in the second dimension. Following staining, each spot represents at least one protein. The same protein may be of various isoforms that differ in charge, but not in mass due to post-translational modifications. A protein spot can be excised from the gel for identification by mass spectrometry as illustrated in Figure 3.

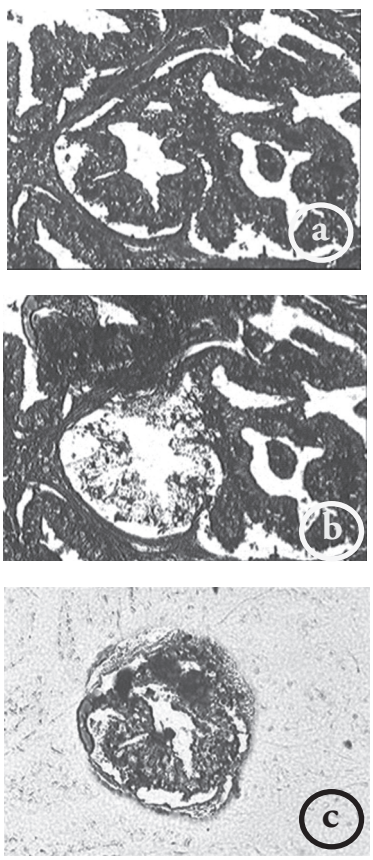

Figure 2 - Precise procurement of cell populations by LCM. **a)** Prostatic glandular epithelial cells in an ethanol-fixed, paraffin-embedded tissue section are imaged before isolation; **b)** Some of these cells are procured with high accuracy and specificity; **c)** the remaining of the tissue following procurement is intact and neighboring cells can be procured for comparative analysis.

them via a stream of electrical fields according to their mass/charge (m/z) ratio. Mass spectrometry instruments are composed of 3 components: an ionization source, a mass analyzer, and an ion detector. Recent and continuing development and improvement of these instruments have revolutionized the field of proteomics. In particular, a number of modifications have enabled high-throughput analysis of protein samples including the invention of soft ionization methods, the innovation of hybrid instruments composed of 2 mass analyzers, and the direct integration of liquid chromatography to MS instruments.

Prior to protein analysis by MS, the protein is specifically digested into smaller peptide fragments by a protease, preferably trypsin. The sample is then introduced into the mass spectrometer for ionization. There are 2 common types of "soft" ionization methods; the first is termed as Matrix-Assisted Laser Desorption/ Ionization (MALDI), where samples are embedded into specific matrix molecules. The matrix absorbs the ionization laser beam and transfers the energy into the analyte. The second ionization method was termed as electrospray ionization (ESI) in which peptides are injected into the ionizing chamber to be converted into smaller droplets. These droplets are vaporized in the presence of an electric field creating charged molecules. Once ionized, molecules are directed via a mass analyzer towards a detector. Whereas ESI requires the peptides be in liquid state and generates ions of multiple charges, MALDI can handle peptides in gaseous, liquid, or solid state and generates ions of similar amounts of energy. In addition, MALDI is more tolerant than ESI to contaminants such as salts and non-ionic detergents.

In addition to being classified according to the ionization method, namely ESI or MALDI, MS instruments can be classified according to their component of mass analyzer. Among the most commonly used analyzers are ion trap (IT), quadrupole (Qq), Fourier-transform ion cyclotron resonance (FTICR), and time-of-flight (TOF). In TOF, the ions are accelerated from the ionization source down a flight tube until they impact the ion detector at the other end of the tube. Since all ions carry the same amount of energy but have different masses, smaller ions travel faster and reach the detector earlier than larger ones. Based on the time needed for the ion to reach the detector, the masses on ions can be calculated generating a "peptide mass fingerprint" (PMF) of the analyzed protein. Time-of-flight mass analyzer is commonly combined with MALDI instruments, hence known as MALDI-TOF.

Electrospray ionization is usually interfaced to other mass analyzers including ion trap, quadrupole, and FTICR. Quadrupole mass analyzers are composed of 4 parallel rods generating an electrical field that focuses the ions and sorts them according to their m/z. In ion trap analyzers, ions are trapped in a 3-dimensional electrical field for selective isolation. Fourier-transform ion cyclotron resonance is a special type of ion traps except that ions are trapped in a magnetic field rather than an electrical one. Fourier-transform ion cyclotron resonance is a powerful mass analyzer providing the highest sensitivity, resolution and mass accuracy. For example, it has been reported that FTICR-MS can identify peptides as low as zeptomoles (10-21 moles).[26]

Since proteins would differ in their proteolytic digestion generating unique PMF, the protein identity can be determined. This is carried out by comparing the peptides' mass spectra of the analyzed protein to theoretical mass spectra of other proteins in a sequence

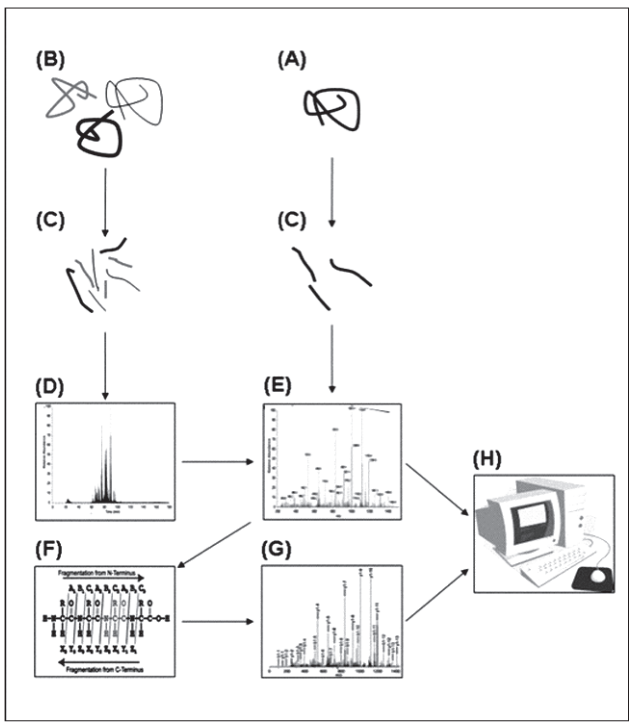

Figure 3 - An outline of protein identification by mass spectrometer (MS). Either a single protein (A) or a collection of proteins (B) can be identified. Proteins are digested into smaller peptides by a specific protease (C). If the sample is complex, peptides are fractionated by liquid chromatography (D), and injected directly into ionizing source of the mass spectrometer. Otherwise, a simple sample containing peptides of an individual protein (A) can be injected directly into the mass spectrometer. Following peptide ionization, ions travel through the mass analyzer generating a peptide mass fingerprint (E). For more accurate identification of proteins by MS-MS instruments, selective peptide ions are directed into a collision-induced dissociation (CID) chamber where they are fragmented into smaller ions (F). These ions are analyzed by a second mass analyzer generating mass spectra specific for that peptide (G). Both peptide mass fingerprints generated (E and G) are interpreted by computational methods (H) in order to determine the protein identity.

database. However, PMF only is not sufficient to identify a protein in cases when a complete protein database is not accessible, multiple proteins from the same family or protein isoforms are present, the mass spectrometer is not of adequate mass accuracy or resolution, or supplementary information such as approximate molecular weight and isoelectric point (from 2D-PAGE, for example) is not available. The advent of tandem mass spectrometry (MS/MS) instruments has significantly transformed these instruments allowing for determination of the amino acid sequences of peptides. These instruments are composed of 2 mass analyzers. Following measurement of peptide masses by the first mass analyzer, few peptide ions are individually selected, fragmented by collision-induced dissociation (CID) yielding smaller ions, and analyzed further by a second mass analyzer. The dual mass analysis leads to determination of the amino acid sequence of the peptides resulting in more accurate identification of proteins. Hybrid MS instruments include innovative combinations of mass analyzers, which can be of the same or different type, such as MALDI TOF-TOF (the first and second mass analyzers are TOF), MALDI–Qq-TOF (the first mass analyzer is quadrupole and the second is TOF), and so forth. **Figure 3** illustrates the mechanism of MS and MS/MS as an example of MS instruments. For more information on concept of MS and the different types of MS instruments, refer to Hirsch et al.[27] and Domon and Aebersold.[28]

Integration of liquid chromatography with MS (LC-MS) has added much needed flexibility in protein identification. Instead of directly injecting a few peptides into the MS, it is possible to analyze a sample composed of millions of peptides simultaneously separated by reverse-phase LC connected to MS, mainly ESI-type. As peptides are separated by LC, they are injected directly into mass spectrometer. This is referred to as "shotgun proteomics".[29-31] This method overcomes the problem associated with 2D-PAGE in identification of hydrophobic or basic proteins. Yates et al[32] have taken the LC-MS integrative process a step further by introducing a technique called MudPIt, or Multi-dimensional protein identification technology, where peptides are separated by 2 chromatography systems (cation followed by reverse-phase LC) leading to the identification of more proteins than a one-dimensional LC-MS.[32] An additional improvement of MS techniques includes the ability to perform differential quantitative protein profiling where 2 samples can be labeled differently, combined, and analyzed simultaneously in search for quantitative differences in protein expression.[33,34]

Surface enhanced laser desorption ionization (SELDI). Surface enhanced laser desorption ionization is a new technology that is a modification of MALDI-TOF mass spectrometry.[35] A major difference is that a sample is applied on surface of a chip rather than mixed with a matrix molecule. The chip surface is made of a defined chemical property (such as hydrophobic, cationic, and anionic) allowing certain classes of proteins to adsorb. The chip is then placed in a vacuum chamber of the SELDI instrument where proteins and peptides are ionized and travel towards a detector inversely according to their masses. The time-of-flight of ions, in particular of peptides below 20 kDa, can be viewed as MS spectra, a chromatogram, of gel-like bands (**Figure 4**).

Due to its speed, high sensitivity, ease of use, and reproducibility, SELDI is a true high throughput proteomic instrument. Two major advantages of SELDI is the ability to analyze highly complex samples, and the low volume of needed for analysis, which can be as low as 0.5 µl. SELDI is also versatile where, instead of a chemical surface, the chip can be coated with antibodies to capture specific antigens has been reported earlier in measuring prostate-specific antigen and prostate-specific membrane antigen.[36-38]

Surface enhanced laser desorption ionization has also been utilized in search for biomarkers for Alzheimer's disease[39,40] as well as cancers of the prostate,[41-43] bladder,[44] colon,[45] and breast.[46] Although direct determination of proteins represented as mass peaks is not possible, different means can be utilized to reveal the identity of specific peaks. In a recent report, SELDI analysis of cerebrospinal fluid (CSF) samples of patients with multiple sclerosis revealed the presence of a differential

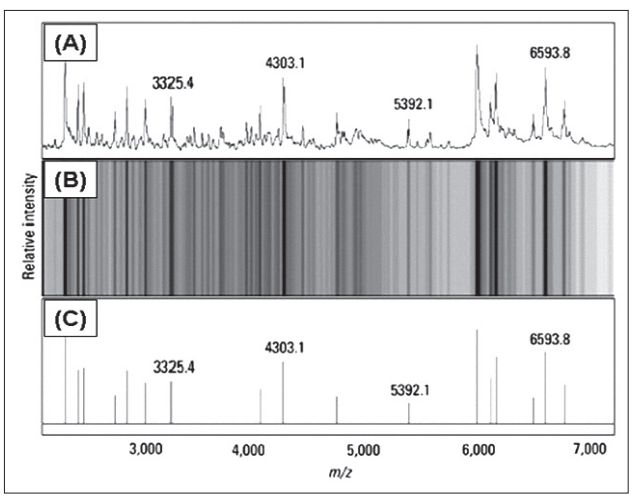

Figure 4 - Representation of protein profiling by surface enhanced laser desorption ionization (SELDI). Measurement of protein time-of-flight can be illustrated graphically as a chromatogram peaks (A), gel-like bands (B), or a mass spectra (C) with every peak, band, or spectrum correspond to a protein.

peak when compared to subjects with other diseases.[47] A differential peak was observed between control and multiple sclerosis samples. This protein was identified by further MS analyses as cystatin C, an inhibitor of the lysosomal cysteine protease cathepsin B. Although burdensome and elaborate, proteins represented by specific SELDI spectra peaks can be identified by a series of liquid chromatography fractionation has been illustrated by Diamond et al,[48] Sanchez et al,[49] and Yang et al.[50]

In one study, proteins extracted from LCM-microdissected prostatic normal and tumor cells were analyzed by SELDI. The mass spectra patterns of the proteins revealed several remarkable alterations as compared to those of matched normal samples.[51] However, due to the dynamic heterogeneity of proteomes even within the same individual, consistent detection of differential peaks is not feasible. This complexity has prompted the group of Petricoin and Liotta to integrate an artificial neural network algorithm to search for "hidden" patterns. In a fascinating study, they have been able to differentiate ovarian cancer patients from normal subjects and patients with other ovarian diseases with unprecedented sensitivity of 100% and specificity of 95%.[52] These results are significant accomplishment considering that: first, the 5-year survival rate is 11% for stage IV ovarian cancer patients in comparison to up to 93% for early-stage patients, and second, there are no superior biomarkers for detecting early-stage ovarian tumors, and, therefore, two-third of ovarian cancers are detected in advanced stage.[53] Very importantly, the SELDI analysis is performed using a small volume (a few microliters) of unfractionated serum samples. The latter study has initiated similar studies on numerous diseases with promising findings in detecting a variety of disorders.[54-57]

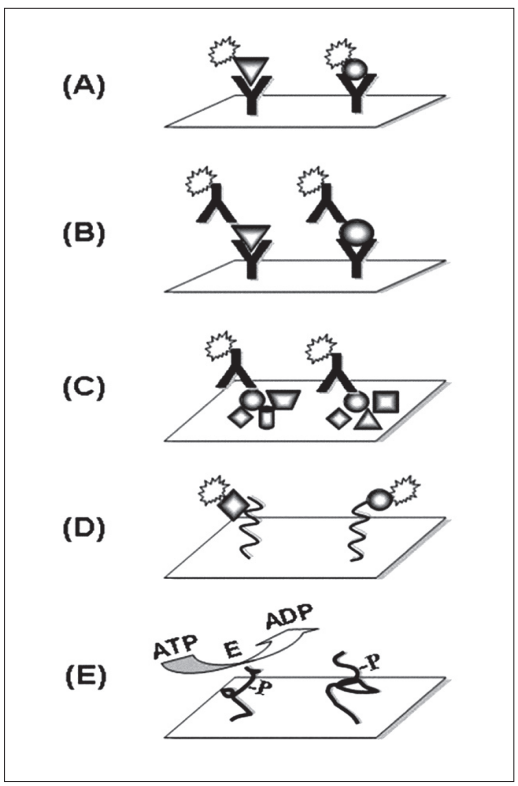

Figure 5 - Protein microarray assay formats. (A) and (B) represent a forward-phase antibody microarrays for measuring protein abundance. In (A) a labeled sample is added onto antibody-arrayed slide, whereas in (B) bound proteins are detected by a second, labeled antibody. (C) is a representation of a reverse-phase protein microarray where complex analytes are spotted onto a slide and specific proteins are detected by a labeled antibody. (D) and (E) are examples of functional protein microarrays where protein binding to spotted DNA fragments (D) can be analyzed. Otherwise, the ability of enzymes, E, to modify arrayed peptides (E) can be tested.

Protein microarray. The success of DNA microarrays has encouraged scientists to invent a similar technology for proteins. Such technology, known as protein microarrays, would allow high-throughput, high sensitivity, and robust analysis of thousands of proteins. Different types of protein microarrays have been designed and can be categorized according to their end-point purpose (**Figure 5**). The first most common design is termed expression or abundance–based protein microarrays. This type of microarrays entails spotting thousands of bait molecules on a glass or membrane-coated slide. Each spot would then represent specific bait for a single protein. Usually the bait molecule is an antibody,[58] although other capture molecules such as aptamers (small DNA or RNA molecules) or phage lysates can be used.[59,60] By incubating a sample containing mixed populations of proteins onto the spotted slides, protein molecules would bind specifically to the corresponding bait molecule. Captured proteins can be detected by directly labeling the sample before applying them onto the slide. This direct labeling method has been utilized in identifying biomarkers of prostate cancer[61] and radiation-regulated proteins.[62] With the direct labeling method, it is possible to analyze more than one sample each labeled with a different fluorescent tag.[63] Otherwise, an indirect labeling method, also known as sandwich immunoassay, can be used where bound proteins are targeted by a second bait such as a different primary antibody that targets a different domain as has been illustrated in measuring the expression the epidermal growth factor receptor and ERB2 and monitoring EGF-dependent phosphorylation in human tumor cells.[64]

Both direct and indirect labeling expression microarrays are also known as forward-phase protein

microarrays. They are in contrast to another design of expression microarray technology termed "reverse-phase protein microarrays".[41] This technology involves spotting the analytes (namely protein extracts) rather than bait molecules, with each spot representing a single test sample. Lysates can be spotted at different dilutions providing an internal standard curve and an opportunity for quantitative measurement. It is worthy to mention that spotted lysates can be obtained from LCM-microdissected cells allowing for studies of pure cell populations. For example, differential protein expression in microdissected prostate cancer cells has been compared to that in patient-matched normal and premalignant cells from the same tissue samples.[41] In addition to expression pattern, signal transduction circuitry can be studied using reverse-phase protein microarrays.[41,65,66] Whereas with forward-phase proteins microarrays, one can analyze multiple proteins in a maximum of 2 samples per one microarray, reverse-phase protein microarrays enable investigation of one or 2 proteins in multiple samples per one microarray. Both types of expression protein microarrays are hampered, though, by the availability of a specific bait molecule (antibody, for example).

Since a major goal of proteomics is to assign function to proteins, 2 other types of protein microarrays have been developed: functional microarrays and interaction microarrays. The enzymatic activities of purified 119 yeast proteins predicted to be tyrosine kinases have been investigated using a functional microarray where kinase substrates are arrayed on a solid surface.[67] Purified proteins are then added individually to the microarrays in the presence of Pγ-ATP.[32] The ability of 27 of the 119 proteins to phosphorylate certain substrates has confirmed their kinase activity. In addition, effects of DNA mutations and polymorphism on DNA-protein binding have been studied using interaction protein microarrays.[68] Interaction arrays can be designed to investigate interaction of certain proteins to various types of molecules including other proteins, peptides, nucleic acids, lipids, carbohydrates, and small molecule.[69-74]

The future. Despite the many challenges, it is certainly an exciting time, the scientific research is going through. Completion of the Human Genome Project has surely been a major factor in increasing understanding of many aspects of biological systems. Similarly, knowledge of human proteomes will contribute significantly to elucidating the normalcy as well as the malfunctions of our genetic and proteomic makeup. It is, however, integration of genomic and proteomic information that will offer a clearer picture of the human biological system. This knowledge will help us understand the physiological and pathological conditions and, hence, the designs are safer and more specific therapeutics. We hoped that we achieve the level of personalized medicine, where therapy is tailored according to the genetic and proteomic makeup of individuals.[75,76] In order to achieve these goals, stronger interaction and collaboration are needed among clinicians and basic scientists.

References

1. Celis JE, Ostergaard M, Jensen NA. Gromova I, Rasmussen HH, Gromov P. Human and mouse proteomic databases: novel resources in the protein universe. ***FEBS Lett*** 1998; 430: 64-72.
2. Ahram M, Springer DL. Large-scale proteomic analysis of membrane proteins. ***Expert Rev Proteomics*** 2004; 1: 293-302.
3. Emmert-Buck MR, Strausberg RL, Krizman DB, Bonaldo MF, Bonner RF, Bostwick DG, et al. Molecular profiling of clinical tissues specimens: feasibility and applications. ***J Mol Diagn*** 2000: 2: 60-66.
4. Ahram M, Emmert-Buck MR. Approaches to Proteomic Analysis of Human Tumors. In: El-Deiry, WS, editor. Methods in Molecular Biology: Tumor Suppressor Genes: Pathways and Isolation Strategies. Ottowa (NJ): Humana Press; 2003. p. 375-384.
5. Anderson L, Seilhamer J. A comparison of selected mRNA and protein abundances in human liver. ***Electrophoresis*** 1997; 18: 533-537.
6. Ideker T, Thorsson V, Ranish JA, Christmas R, Buhler J, Eng JK, et al. Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. ***Science*** 2001; 292: 929-934.
7. Drews J. Drug discovery: a historical perspective. ***Science*** 2000; 287: 1960-1964.
8. Klose J, Kobalz U. Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. ***Electrophoresis*** 1995; 16: 1034-1059.
9. O'Farrell PZ, Goodman HM. Resolution of simian virus 40 proteins in whole cell extracts by two-dimensional electrophoresis: heterogeneity of the major capsid protein. ***Cell*** 1976; 9: 289-298.
10. Gorg A, Weiss W, Dunn MJ. Current two-dimensional electrophoresis technology for proteomics. ***Proteomics*** 2004; 4: 3665-3685.
11. Gorg A, Obermaier C, Boguth G, Harder A, Scheibe B, Wildgruber R, et al. The current state of two-dimensional electrophoresis with immobilized pH gradients. ***Electrophoresis*** 2000; 21: 1037-1053.
12. Yan JX, Harry RA, Spibey C, Dunn MJ. Postelectrophoretic staining of proteins separated by two-dimensional gel electrophoresis using SYPRO dyes. ***Electrophoresis*** 2000; 21: 3657-3665.
13. Malone JP, Radabaugh MR, Leimgruber RM, Gerstenecker GS. Practical aspects of fluorescent staining for proteomic applications. ***Electrophoresis*** 2001; 22: 919-932.
14. Unlu M, Morgan ME, Minden JS. Difference gel electrophoresis: a single gel method for detecting changes in protein extracts. ***Electrophoresis*** 1997; 18: 2071-2077.

15. Tonge R, Shaw J, Middleton B, Rowlinson R, Rayner S, Young J, et al. Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. ***Proteomics*** 2001; 1: 377-396.
16. Zhou G, Li H, DeCamp D, Chen S, Shu H, Gong Y, et al. 2D differential in-gel electrophoresis for the identification of esophageal scans cell cancer-specific protein markers. ***Mol Cell Proteomics*** 2002; 1: 117-124.
17. Somiari RI, Sullivan A, Russell S, Somiari S, Hu H, Jordan R, et al. High-throughput proteomic analysis of human infiltrating ductal carcinoma of the breast. ***Proteomics*** 2003; 3: 1863-1873.
18. Celis A, Rasmussen HH, Celis P, Basse B, Lauridsen JB, Ratz G, et al. Short-term culturing of low-grade superficial bladder transitional cell carcinomas leads to changes in the expression levels of several proteins involved in key cellular activities. ***Electrophoresis*** 1999; 20: 355-361.
19. Ornstein DK, Gillespie JW, Paweletz CP, Duray PH, Herring J, Vocke CD, et al. Proteomic analysis of laser capture microdissected human prostate cancer and in vitro prostate cell lines. ***Electrophoresis*** 2000; 21: 2235-2242.
20. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, et al. Laser capture microdissection. ***Science*** 1996; 274: 998-1001.
21. Emmert-Buck MR, Gillespie JW, Paweletz CP, Ornstein DK, Basrur V, Appella E, et al. An approach to proteomic analysis of human tumors. ***Mol Carcinog*** 2000; 27: 158-165.
22. Ahram M, Best CJ, Flaig MJ, Gillespie JW, Leiva IM, Chuaqui RF, et al. Proteomic analysis of human prostate cancer. ***Mol Carcinog*** 2002; 33: 9-15.
23. Ahram M, Flaig MJ, Gillespie JW, Duray PH, Linehan WM, Ornstein DK, et al. Evaluation of ethanol-fixed, paraffin-embedded tissues for proteomic applications. ***Proteomics*** 2003; 3: 413-421.
24. Simpson RJ, Connolly LM, Eddes JS, Pereira JJ, Moritz RL, Reid GE. Proteomic analysis of the human colon carcinoma cell line (LIM 1215): development of a membrane protein database. ***Electrophoresis*** 2000; 21: 1707-1732.
25. Adam PJ, Boyd R, Tyson KL, Fletcher GC, Stamps A, Hudson L, et al. Comprehensive proteomic analysis of breast cancer cell membranes reveals unique proteins with potential roles in clinical cancer. ***J Biol Chem*** 2003; 278: 6482-6489.
26. Belov ME, Gorshkov MV, Udseth HR, Anderson GA, Smith RD. Zeptomole-sensitivity electrospray ionization--Fourier transform ion cyclotron resonance mass spectrometry of proteins. ***Anal Chem*** 2000; 72: 2271-2279.
27. Hirsch J, Hansen KC, Burlingame AL, Matthay MA. Proteomics: current techniques and potential applications to lung disease. ***Am J Physiol Lung Cell Mol Physiol*** 2004; 287: L1-23.
28. Domon B, Aebersold R. Mass spectrometry and protein analysis. ***Science*** 2006; 312: 212-217.
29. Wolters DA, Washburn MP, Yates JR 3rd. An automated multidimensional protein identification technology for shotgun proteomics. ***Anal Chem*** 2001; 73: 5683-5690.
30. McDonald WH, Yates JR 3rd. Shotgun proteomics and biomarker discovery. ***Dis Markers*** 2002; 18: 99-105.
31. McDonald WH, Yates JR 3rd. Shotgun proteomics: integrating technologies to answer biological questions. ***Curr Opin Mol Ther*** 2003; 5: 302-309.
32. Washburn MP, Wolters D, Yates JR 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. ***Nat Biotechnol*** 2001; 19: 242-247.
33. Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. ***Nat Biotechnol*** 1999; 17: 994-999.
34. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. ***Mol Cell Proteomics*** 2002; 1: 376-386.
35. Yip TT, Hutchens TW. Mapping and sequence-specific identification of phosphopeptides in unfractionated protein digest mixtures by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. ***FEBS Lett*** 1992; 308: 149-153.
36. Wright GL Jr, Beckett ML, Newhall KR, Adam BL, Cazares LH, Cartwright SL, et al. Identification of a superimmunoglobulin gene family member overexpressed in benign prostatic hyperplasia. ***Prostate*** 2000; 42: 230-238.
37. Xiao Z, Adam BL, Cazares LH, Clements MA, Davis JW, Schellhammer PF, et al. Quantitation of serum prostate-specific membrane antigen by a novel protein biochip immunoassay discriminates benign from malignant prostate disease. ***Cancer Res*** 2001; 61: 6029-6033.
38. Adam BL, Qu Y, Davis JW, Ward MD, Clements MA, Cazares LH, et al. Serum protein fingerprinting coupled with a pattern-matching algorithm distinguishes prostate cancer from benign prostate hyperplasia and healthy men. ***Cancer Res*** 2002; 62: 3609-3614.
39. Austen BM, Frears ER, Davies H. The use of seldi proteinchip arrays to monitor production of Alzheimer's betaamyloid in transfected cells. ***J Pept Sci*** 2000; 6: 459-469.
40. Carrette O, Demalte I, Scherl A, Yalkinoglu O, Corthals G, Burkhard P, et al. A panel of cerebrospinal fluid potential biomarkers for the diagnosis of Alzheimer's disease. ***Proteomics*** 2003; 3: 1486-1494.
41. Paweletz CP, Charboneau L, Bichsel VE, Simone NL, Chen T, Gillespie JW, et al. Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. ***Oncogene*** 2001; 20: 1981-1989.
42. Lehrer S, Roboz J, Ding H, Zhao S, Diamond EJ, Holland JF, et al. Putative protein markers in the sera of men with prostatic neoplasms. ***BJU Int*** 2003; 92: 223-225.
43. Liu AY, Zhang H, Sorensen CM, Diamond DL. Analysis of prostate cancer by proteomics using tissue specimens. ***J Urol*** 2005; 173: 73-78.
44. Vlahou A, Schellhammer PF, Mendrinos S, Patel K, Kondylis FI, Gong L, et al. Development of a novel proteomic approach for the detection of transitional cell carcinoma of the bladder in urine. ***Am J Pathol*** 2001; 158:1491-1502.
45. Engwegen JY, Helgason HH, Cats A, Harris N, Bonfrer JM, Schellens JH, et al. Identification of serum proteins discriminating colorectal cancer patients and healthy controls using surface-enhanced laser desorption ionisation-time of flight mass spectrometry. ***World J Gastroenterol*** 2006; 12: 1536-1544.
46. Ricolleau G, Charbonnel C, Lode L, Loussouarn D, Joalland MP, Bogumil R, et al. Surface-enhanced laser desorption/ionization time of flight mass spectrometry protein profiling identifies ubiquitin and ferritin light chain as prognostic biomarkers in node-negative breast cancer tumors. ***Proteomics*** 2006; 6: 1963-1975.
47. Irani DN, Anderson C, Gundry R, Cotter R, Moore S, Kerr DA, et al. Cleavage of cystatin C in the cerebrospinal fluid of patients with multiple sclerosis. ***Ann Neurol*** 2006; 59: 237-247.

48. Diamond DL, Zhang Y, Gaiger A, Smithgall M, Vedvick TS, Carter D. Use of ProteinChip array surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) to identify thymosin beta-4, a differentially secreted protein from lymphoblastoid cell lines. ***J Am Soc Mass Spectrom*** 2003; 14: 760-765.
49. Sanchez JC, Guillaume E, Lescuyer P, Allard L, Carrette O, Scherl A, et al. Cystatin C as a potential cerebrospinal fluid marker for the diagnosis of Creutzfeldt-Jakob disease. ***Proteomics*** 2004; 4: 2229-2233.
50. Yang EC, Guo J, Diehl G, DeSouza L, Rodrigues MJ, Romaschin AD, et al. Protein expression profiling of endometrial malignancies reveals a new tumor marker: chaperonin 10. ***Proteome Res*** 2004; 3: 636-643.
51. Petricoin EF, Ornstein DK, Paweletz CP, Ardekani A, Hackett PS, Hitt BA, et al. Serum proteomic patterns for detection of prostate cancer. ***J Natl Cancer Inst*** 2002; 94: 1576-1578.
52. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, et al. Use of proteomic patterns in serum to identify ovarian cancer. ***Lancet*** 2002; 359: 572-577.
53. Ozols RF, Rubin SC, Thomas GM, Robboy SJ. Epithelial ovarian cancer In: Hoskins WJ, Perez CA, Young RC, editors. Principles and Practice of Gynecologic Oncology. Philadelphia (PA): Lippincott, Williams & Wilkins; 2000. p. 981–1058.
54. de Torre C, Ying SX, Munson PJ, Meduri GU, Suffredini AF. Proteomic analysis of inflammatory biomarkers in bronchoalveolar lavage. ***Proteomics*** 2006; 66: 3946-3957.
55. Soltys SG, Le QT, Shi G, Tibshirani R, Giaccia AJ, Koong AC. The use of plasma surface-enhanced laser desorption/ionization time-of-flight mass spectrometry proteomic patterns for detection of head and neck squamous cell cancers. ***Clin Cancer Res*** 2004; 10: 4806-4812.
56. Yang SY, Xiao XY, Zhang WG, Zhang LJ, Zhang W, Zhou B, et al. Application of serum SELDI proteomic patterns in diagnosis of lung cancer. ***BMC Cancer*** 2005; 5: 83.
57. Srinivasan R, Daniels J, Fusaro V, Lundqvist A, Killian JK, Geho D, et al. Accurate diagnosis of acute graft-versus-host disease using serum proteomic pattern analysis. ***Exp Hematol*** 2006; 34: 796-801.
58. Haab BB. Advances in protein microarray technology for protein expression and interaction profiling. ***Curr Opin Drug Discov Devel*** 2001; 4: 116-123.
59. Arnaud MC, Gazarian T, Rodriguez YP, Gazarian K, Sakanyan V. Array assessment of phage-displayed peptide mimics of Human Immunodeficiency Virus type 1 gp41 immunodominant epitope: binding to antibodies of infected individuals. ***Proteomics*** 2004; 4: 1959-1964.
60. Choi JK, Yu U, Yoo OJ, Kim S. Differential coexpression analysis using microarray data and its application to human cancer. ***Bioinformatics*** 2005; 21: 4348-4355.
61. Miller JC, Zhou H, Kwekel J, Cavallo R, Burke J, Butler EB, et al. Antibody microarray profiling of human prostate cancer sera: antibody screening and identification of potential biomarkers. ***Proteomics*** 2003; 3: 56-63.
62. Sreekumar A, Nyati MK, Varambally S, Barrette TR, Ghosh D, Lawrence TS, et al. Profiling of cancer cells using protein microarrays: discovery of novel radiation-regulated proteins. ***Cancer Res*** 2001; 61: 7585-7593.
63. Haab BB. Antibody arrays in cancer research. ***Mol Cell Proteomics*** 2005; 4: 377-383.
64. Nielsen UB, Cardone MH, Sinskey AJ, MacBeath G, Sorger PK. Profiling receptor tyrosine kinase activation by using Ab microarrays. ***Proc Natl Acad Sci USA*** 2003; 100: 9330-9335.
65. Nishizuka S, Charboneau L, Young L, Major S, Reinhold WC, Waltham M, et al. Proteomic profiling of the NCI-60 cancer cell lines using new high-density reverse-phase lysate microarrays. ***Proc Natl Acad Sci USA*** 2003; 100: 14229-14234.
66. Petricoin EF, Liotta LA. Clinical applications of proteomics. ***J Nutr*** 2003; 133: 2476S-2484S.
67. Zhu H, Klemic JF, Chang S, Bertone P, Casamayor A, Klemic KG, et al. Analysis of yeast protein kinases using protein chips. ***Nat Genet*** 2000; 26: 283-289.
68. Boutell JM, Hart DJ, Godber BL, Kozlowski RZ, Blackburn JM. Functional protein microarrays for parallel characterisation of p53 mutants. ***Proteomics*** 2004; 4: 1950-1958.
69. MacBeath G, Schreiber SL. Printing proteins as microarrays for high-throughput function determination. ***Science*** 2000; 289: 1760-1763.
70. Ge, H. UPA, a universal protein array system for quantitative detection of protein–protein, protein–DNA, protein–RNA and protein–ligand interactions. ***Nucl Acids Res*** 2000; 28: e3.
71. Iyer VR, Horak CE, Scafe CS, Botstein D, Snyder M, Brown PO. Genomic binding sites of the yeast cell-cycle transcription factors SBF and MBF. ***Nature*** 2001; 409: 533-538.
72. Zhu H, Bilgin M, Bangham R, Hall D, Casamayor A, Bertone P, et al. Global analysis of protein activities using proteome chips. ***Science*** 2001; 293: 2101-2105.
73. Linnell J, Groeger G, Hassan AB. Real time kinetics of insulin-like growth factor II (IGF-II) interaction with the IGF-II/mannose 6-phosphate receptor: the effects of domain 13 and pH. ***J Biol Chem*** 2001; 276: 23986-23991.
74. Espejo A, Cote J, Bednarek A, Richard S, Bedford MT. A protein-domain microarray identifies novel protein-protein interactions. ***Biochem J*** 2002; 367: 697-702.
75. Liotta LA, Kohn EC, Petricoin EF. Clinical proteomics: personalized molecular medicine. ***JAMA*** 2001; 286: 2211-2214.
76. Jain KK. Personalized medicine. ***Curr Opin Mol Ther*** 2002; 4: 548-558.