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REVIEW ARTICLE

Clinical proteomics: Current status, challenges, and future perspectives

臨床蛋白質體學：目前現況，挑戰與未來展望

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關鍵詞
一維或二維凝膠電泳;
液相層析串聯質譜儀;

Abstract This account will give an overview and evaluation of the current advances in mass spectrometry (MS)-based proteomics platforms and technology. A general review of some background information concerning the application of these methods in the characterization of molecular sizes and related protein expression profiles associated with different types of cells under varied experimental conditions will be presented. It is intended to provide a concise and succinct overview to those clinical researchers first exposed to this foremost powerful methodology in modern life sciences of postgenomic era. Proteomic characterization using highly sophisticated and expensive instrumentation of MS has been used to characterize biological samples of complex protein mixtures with vastly different protein structure and composition. These systems are then used to highlight the versatility and potential of the MS-based proteomic strategies for facilitating protein expression analysis of various disease-related organisms or tissues of interest. Major MS-based strategies reviewed herein include (1) matrix-assisted laser desorption ionization-MS and electron-spray ionization proteomics; (2) one-dimensional or two-dimensional gel-based proteomics; (3) gel-free shotgun proteomics in conjunction with liquid chromatography/tandem MS; (4) Multiple reaction monitoring coupled tandem MS quantitative proteomics and; (5) Phosphoproteomics based on immobilized metal affinity chromatography and liquid chromatography-MS/MS.

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串聯質譜多重反應監控掃瞄;
固定化金屬親和層析;
磷酸化蛋白質體學

摘要 本評論文章將針對以質譜為基礎的蛋白質體學平台和技術的進展做一現況回顧和評價。文章中將廣泛性回顧過去應用質譜方法,分析鑑定來自各種實驗條件下不同來源細胞的蛋白質分子大小與整體蛋白質表現圖譜。本文章的目的是針對首次接觸到這個最重要且功能強大的質譜學新技術方法的臨床研究人員,在後基因體時代應該具備的簡明基礎性蛋白質體學概念。運用高度複雜和昂貴質譜儀器的蛋白質體鑑定技術,已被用於鑑定具有截然不同的結構和組成的複雜蛋白質混合物生物樣品。這些顯著且具有多功能性和有潛力性的系統則已被有效應用於各種疾病相關的微生物或組織的蛋白質體表達分析。這些以質譜為基礎的主要策略回顧包括：1.基質輔助雷射脫附游離質譜和電灑游離的蛋白質體學 2.一維或二維凝膠電泳的蛋白質體學 3.無凝膠散彈槍法蛋白質體學連接液相層析/串聯質譜（LC-MS/MS）4.多重反應監控掃瞄耦合串聯質譜（MS/MS-MRM）的定量蛋白質體學 5.利用固定化金屬親和層析（IMAC）結合液相層析串聯質譜的磷酸化蛋白質體學。

Introduction and background

Modern clinical research is increasingly dependent on evidence-based approach to biomedical research issues related to human diseases with the advent of postgenomic era and the completion of human genome sequences. There comes next the functional genomics with its major emphasis on interactions between various genome-encoded proteins. Subsequently, proteomics in the context of the link between genes and proteins arose and evolved in robust speed to become a major theme of central topics in modern biomedical research.

Proteomes depict snapshots of protein compositions of particular cells or tissues at defined time points. Through the assistance of constantly increasing databases of complete genomes from various model organisms, including human and various submammalian species [1], proteomes of any samples from some exotic biological sources can now be analyzed at protein expression levels by various separation methods coupled with mass spectrometry (MS) regarding their protein identities in relation to other known protein sequences deposited in various databanks. To date, proteomics through the fast-evolving MS instrumentation has been considered probably the most powerful and enlightening breakthrough to be reckoned with in the research realm of biomedical science. Recently, clinician scientists who have gradually witnessed and appreciated the epoch-making potential and impact of proteomics to biotechnology and medicine in the foreseeable future began to use innovative proteomic technology to clinical research especially on its application to translational medicine. They are especially interested in coupling the proteomics technology with bioinformatics mining of human genome to unravel the related molecular mechanisms of diseases. This has indeed engendered the advent of clinical proteomics [2—12]. The newly developed discipline would focus on effective clinical monitoring and diagnosis of patients before and after medical treatment and the prognosis and assessment during the progress of disease treatment. This usually involves several phases of proteomic study, that is, (1) biomarker discovery; (2) biomarker verification; and finally (3) biomarker validation and clinical trial.

In this review, we have made an endeavor to summarize and compare the following widely used workflow and methodological strategies (Fig. 1) for the holistic proteomic approach to characterize protein expression profiles of various disease-related organisms or tissues of interest with the aim of providing some application guidelines to clinical researchers interested in using clinical proteomics to translational medicine.

Proteomics strategies and platforms

Matrix-assisted laser desorption ionization-MS and electron-spray ionization proteomics

Tanaka et al. [13] reported the first mass spectra for several large protein molecules in 1988 by using nanometal particles-assisted laser desorption, which started the macromolecular characterization based on MS measurement for biomolecules in ionized states. At about the same time, Karas and Hillenkamp [14] also developed matrix-assisted laser desorption/ionization (MALDI) MS for measurement of molecular masses of different proteins with a time-of-flight (TOF) MS. On the other hand, Fenn and co-workers [15] developed electrospray ionization (ESI)-MS, which can also give soft ionization of proteins. It is to be noted that MS developers have tried to put major efforts in developing MSs for macromolecule detection for many decades in vain mainly because of the difficulty involved in the ionization of macromolecules in vacuum to various ionized states. Nowadays, MALDI and ESI MSs have overcome the ionization problem to become the two major tools of choice for proteomic study of complex biological systems especially protein and peptide analysis, for which Tanaka and Fenn won the Nobel Prize in 2002.

In general, the mass resolution and accuracy of a MALDI-TOF MS is not high enough to give a nonambiguous identification of a peptide with high confidence. Moreover, some amino acid residues have similar or even identical molecular weights (MWs). For example, the masses of both isomeric amino acids, that is, isoleucine and leucine are 113.16 daltons (Da). The molecular masses of glutamine and lysine are 128.131 and 128.174 Da, with difference between these the two being within 0.043 Da. Therefore, it is not feasible to use MALDI-TOF for deriving protein sequences unambiguously by measuring masses of various enzyme-digested fragments to be analyzed in this type of MS instrument. On the other hand, MALDI-TOF and its variant surface-enhanced laser desorption/ionization(SELDI)-TOF possess unique advantages of enabling a high-throughput analysis for proteomic studies of multiple samples. These two MS platforms have been commonly used to study expression proteomics (i.e. protein profiling) of different types of tissues obtained from the cohort patients. To date, MALDI ionization mechanism is still not well understood. It can be essential to learn

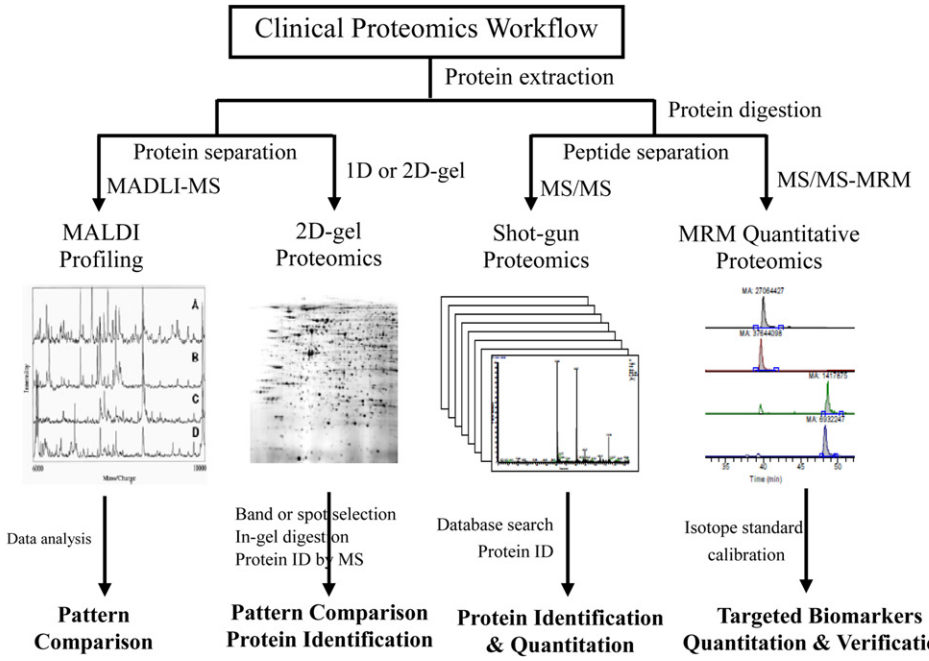

Figure 1. Scheme of the current workflow and methodological strategies for proteomics application in biological science and medicine. (Adapted and modified from: http://fenchurch.mc.vanderbilt.edu/lab/bmif310/2007/4-F-Liebler-Clinical-Proteomics.pdf). MALDI-MS = matrix-assisted laser desorption ionization mass spectrometer; MRM = multiple reaction monitoring; protein ID = protein identification.

more on the ionization mechanism to further improve MS for biomolecular detection. Nevertheless, MALDI has been quite efficient in detecting peptides (~1 fmole) but less successful in oligonucleotide and polysaccharide especially when the size is larger than 100,000 Da [1].

Collision-induced dissociation (CID) was introduced in 1968 to obtain structure information with a tandem MS [16,17]. The first mass analyzer is used to determine the mass spectrum of the sample and the second mass spectrum (denoted as MS/MS or MS^2) is used to determine the structure of selected peaks from the first mass spectrum by a collision process with selected gas molecules. Sometimes, higher orders of mass spectra (MS^n) induced by CID can be obtained to get more information on the identification of biomolecular structures. To meet the demand of high-quality proteomic studies, CID has been quickly adopted by proteomic community to give more reliable determination of sequences of peptides, which can be subsequently used for more accurate protein determination.

Currently, there are two fundamental strategies for proteomics study. One is bottom-up and the other is top-down. In bottom-up approach, purified proteins or complex protein mixtures are subjected to chemical or enzymatic cleavage and the peptide products are usually separated by chromatography followed by mass spectrometric analysis [18]. In top-down proteomics, intact protein ions or large protein fragments are subjected to gas-phase fragmentation for MS analysis directly [19–21]. With top-down analysis, all post-translational modifications (PTMs) will be subjected to analysis, whereas bottom-up analysis may skip the fragments with PTM. Because many fragmentation processes, such as CID are not efficient for very large proteins (MW > 100,000 Da) in routine operation, a true top-down strategy works only for relatively small proteins. Some researchers also considered MS analysis of peptides obtained from *in situ* digestion of proteins after two-dimensional (2D) gel separation as a top-down strategy (see below). With rapid progress in MS technology development and bioinformatics during the past few years, proteomics study to identify various proteins in complex protein mixtures isolated from tissues or cells of interest is expected to become a routine high-throughput exercise in the near future.

On the other hand, the special advantages for ESI as compared with MALDI instrumentation include: (1) high reproducibility: no crystallization process for sample preparation is involved; (2) high flexibility to attach to different types of MS: the ESI source with lower mass-to-charge ratios (m/z ratios) can be fitted to ion-trap, quadrupole, Fourier-transform ion cyclotron resonance and TOF-MS because of ESI's preference of multiple charged ions generated from various biomolecules. The major disadvantages are (1) complex spectra because of peaks from multiply charged ions; (2) large sample quantity: this disadvantage more or less disappears after the introduction of nanospray [22]; (3) ESI cannot be used for molecular imaging. The recent development of desorption ESI nevertheless can compensate this defect, which can be applied for molecular imaging but the space resolution is still significantly worse than that obtained by MALDI [1].

1D or 2D gel-based proteomics

In 1993, Henzel et al. [23] reported the first study related to the global identification of proteins in biological samples based on preseparation of protein mixture on 2D gel electrophoresis (2-DE). The peptides were generated by *in situ* tryptic digestion of proteins on polyacrylamide gels. Masses of different peptides were analyzed by MALDI-TOF

MS. The mass spectral patterns of protein fragments generated with trypsin digestion were then used for comparison with those of previously known proteins deposited in databanks to confirm peptides, which can be used for identification of intact proteins (protein ID). This study actually started and played a major role in the popular and widespread proteomics analysis by combining gel electrophoresis and MS.

Conventional protein separation and analysis by gel electrophoresis started with one-dimensional polyacrylamide gel electrophoresis (1D gel or 1D PAGE). The limitation for the analysis of complex protein mixtures is that closely spaced protein bands tend to overlap, so that any 1D gel separation method, such as sodium dodecyl sulfate (SDS)-PAGE (PAGE in the denaturing agent SDS) can only resolve only a relatively small number of proteins (generally fewer than 50) [24]. In 1975, O'Farrell [25] developed a new method of 2D PAGE to overcome the shortcomings of 1D PAGE. Combining the features of two different separation procedures with the first dimensional separation being based on isoelectric points (pI) and the second dimensional separation on molecular sizes of protein components in the samples, 2D PAGE can resolve more than 1,000 different proteins in the form of a 2D polypeptide map [25]. In the first dimension, the sample of interest is dissolved in a small volume of a solution containing a non-ionic detergent (e.g. 0.5% (w/v) NP-40), the denaturing reagent urea, and reducing agents, such as dithiothreitol or mercaptoethanol. This sample buffer solution can dissolve and dissociate most of the proteins (with some exceptions) from any tissues without changing their intrinsic charges. Then the polypeptide mixture is separated by the principle of isoelectric focusing. In the second step, the narrow gel containing the separated proteins is soaked in SDS and the proteins are further fractionated according to the size by SDS-PAGE on a slab gel. Each polypeptide chain now migrates as a discrete spot on the gel according to its MW. The only proteins left unresolved will be those that have both an identical size and an identical pI, a relatively rare situation. There has been an increasing emphasis on the separation and characterization of polypeptides by 2D PAGE in recent years, which has been claimed to be the most sensitive and high-resolution method for the analysis of protein composition in any cell extract or protein mixture. The second dimension of SDS-PAGE can usually be run in gradient gels [24] to even improve the resolution. For higher sensitivity, cell extract or protein mixture can be labeled with a radioisotope before running 2D PAGE. Up to 2,000 individual polypeptide chains can be resolved on a single 2D gel, enough to account for most of the proteins in a bacterium. One reason for the popularity of this method is that because the two dimensions separate proteins on the basis of two independent parameters (their pIs in one dimension and protein subunit sizes in the other), one usually obtains an excellent resolution of even very complex protein mixtures. A 2-DE was thus considered as the method of choice, as it could afford a high throughput and relatively high-resolution analytical tool to resolve and separate a mixture of thousands of protein species with different charge and size properties [24]. However, the serious drawback of low sensitivity can be attributed to the fact that the maximal numbers of several thousands of spots resolved and analyzed by 2D is still relatively small as compared with the whole genome-encoded functional proteins of about 20,000—30,000 proteins, especially being underrepresentative of some special classes of proteins, such as the extremely basic or acidic groups of proteins, some low-abundance transcription factors, and membrane proteins [24,26,27] because of the low solubility of these classes of proteins in the first dimensional isoelectric focusing gel. Currently, 1-DE instead of 2-DE in the presence of the most effective denaturing agent of SDS is more often used for proteomic analysis of samples of poor solubility. A proteomic sample is first separated and resolved in the 1D gel (Fig. 2A). The proteins can usually be separated into at least 5 to more than 10 different blocks depending on the complexity of the proteomic samples. Then the separated protein bands are subjected to digestion with trypsin. The products are subsequently fed into a liquid chromatograph MS (LC-MS or LC-MS/MS) for protein ID. Fig. 2A and B showed the representative 1DE and 2DE of protein mixtures of human and porcine eye lenses under dissociating conditions in the presence of SDS for the resolution of complex protein components. These were carried out on polyacrylamide gels using slab gel instead of rod gel systems. It can be seen that 1D gel being less tedious and time consuming than 2D gel still affords a respectable protein separation and protein ID analysis similar to that of 2D gel.

Gel-free shotgun proteomics in conjunction with LC-tandem MS (LC-MS/MS)

The recent explosion in available genomic sequence information is providing a useful sequence infrastructure for clinical proteomics. A major aspect of various proteomics strategies is the protein ID using analytical "fingerprints" or peptide mass fingerprinting (PMF) generated by digestion of proteins with specific enzymes, such as trypsin, followed by tandem mass (MS/MS) spectrometry, from which MS/MS spectra of peptide fragments can then be used for comparison and search in available sequence databanks.

The mixture of enzyme-digested proteins or peptides can usually be separated by multidimensional LC (MDLC) and analyzed by a tandem MS. Various MS/MS spectra can be algorithmically compared with predicted peptide spectra from sequence databases to identify the respective proteins. The digestion of intact protein or peptide mixtures with various enzymes of different specificities followed by the direct analysis of the resulting peptides by high-resolution LCs coupled with tandem MS/MS has facilitated the so-called "shotgun" strategy for the identification of protein mixtures without the need for prior sample fractionation based on 1D or 2D gels (gel-free proteomics). By combining with the recent development of capillary MDLC, this approach is now capable of characterizing proteins directly from entire cell lysates [28—31]. In shotgun proteomics, MDLC is a necessity to reduce sample complexity and increase dynamic range of protein identification. For some years shotgun proteomics has been the method of choice for analysis of complex samples because of its ease of automation, high throughput, and sensitivity. Shotgun proteomic approach involving nano-LC quadrupole-TOF MS has also been especially attractive for efficient

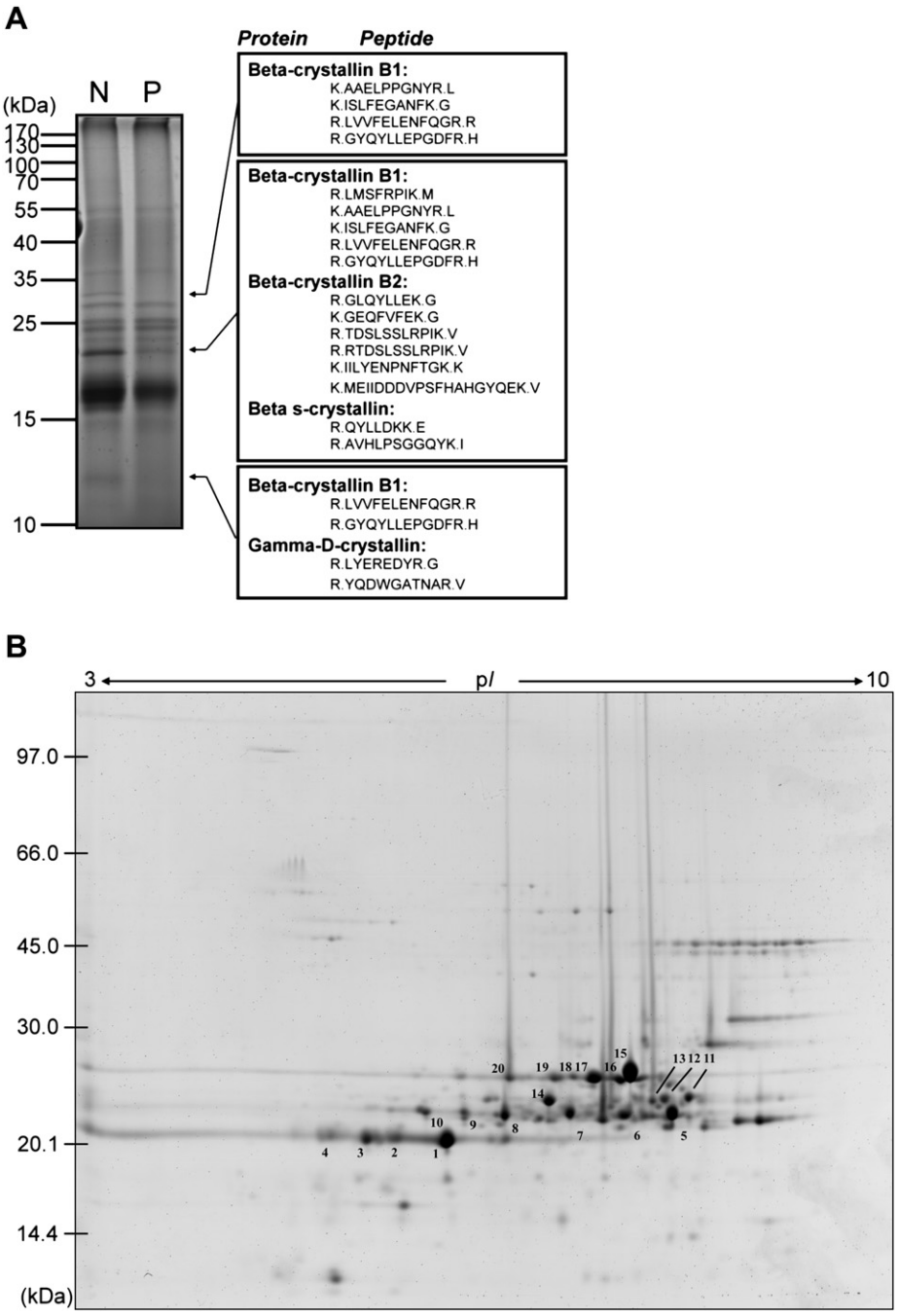

Figure 2. Analysis of lens proteins by 1-DE and 2-DE gel electrophoresis. (A) Analysis of lens proteins from normal human lens without cataract by SDS-PAGE. As shown in the left panel, a total of 10 μg lens proteins were separated and resolved in 12.5% SDS-PAGE and stained with Coomassie brilliant blue R-250. N, normal person; P, cataract patient. In the right panel, protein bands (indicated by arrows) were in-gel digested with trypsin, followed by LC-MS/MS to find protein ID based on PMF of digested proteins. (B) Two-DE gel patterns of porcine lens proteins. 100 μg total protein was loaded onto immobilized pH gradient (IPG) gel strips (pH 3—10 nonlinear, 13 cm). For the 1D separation, IEF was carried out at 300—8,000 V for 16 hour. After IEF, the IPG strips were equilibrated in SDS-urea buffer and placed onto the 2D SDS-PAGE. After electrophoresis, the gels were fixed in 10% methanol and 7% acetic acid and stained by Sypro-Ruby. Protein spots marked by No. 1—20 on the map were further identified by nano LC-MS/MS. The result is representative of three independent experiments. (Courtesy of Molecular Vision Press; [63]). 1-DE = one-dimensional gel electrophoresis; 2-DE = two-dimensional gel electrophoresis; IEF = isoelectric focusing; LC-MS = liquid chromatography coupled tandem mass spectrometry; pI = isoelectric points; PMF = peptide mass fingerprinting; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

proteomic analysis of limited and scarce samples. Recently mass spectrometric methods are being developed along the line that not only identifies proteins in a mixture but also compares the relative levels of protein expression between two different samples, that is, quantitative proteomics.

Quantitative proteomics

To date, the current bottom-up proteomic approach to find protein ID in various biological samples gradually becomes a routine practice. Now the major challenge lies in quantitative measurements for different proteins in unknown samples of interest. Biomarker discovery is especially dependent on the accurate quantitative difference between normal and diseased samples. In the search for disease-associated protein determinants, reproducible and quantitative proteomic analysis remains a vital analytical part in the study of the translational effects of various protein biomarkers in regulating cellular activity and function. Therefore, quantitative determination of each individual protein deserves more efforts. On the other hand, the extension of dynamic range to measure ultra-low quantity of proteins inside a proteomic sample remains a high priority and a great demand. Currently, there are two different approaches to quantitative proteomics. One is strictly based on mass spectra from MS/MS without labeling and the other relies on the labeling methodology.

Label-free quantitative proteomics

Label-free approach for quantitative proteomics is more attractive because it can reduce the contaminant species during the labeling process before proteomic analysis with high sensitivity. Nevertheless, the entire process for proteomic analysis without a quantitative labeling tag is quite complex. It is also very difficult to assure quality control on every purification and analytical step in different laboratories or different personnel from the same laboratory. For example, sample collection can be a big concern. It is known that degradation of proteins/peptides can occur without proper precaution. Ionization efficiency for a selected protein under a different environment can be quite different in both MALDI and ESI ionization processes. It is well known that ionization is a strong function of acidity in the sample. When LC-MS or MDLC-MS on-line analysis is used, the quantity of proteins/peptides to be analyzed can also depend on the time protein/peptide is eluted for MS analysis. Owing to the great advantages of simplicity and time saving, tremendous effort has been placed on developing label-free quantitative proteomics. In general, for label-free LC-MS experiments to achieve quantitative determination, peptide ion intensity counting and spectral counting have been used extensively. At present, there are still controversies over the reliability of label-free quantitative analysis. It is noteworthy that quantitative determination between two samples cannot be just based on qualitative protein ID analysis without a dedicated proteomic analysis platform to do data analysis. Heavy participation of bioinformatics software analysis is warranted for the future success of label-free quantitative analysis.

Label-based quantitative proteomics

Isotopic-coded affinity tag

The basic principle of Isotopic-coded affinity tag (ICAT) [32] relies on the protein thiol-specific reagents. It consists of three major steps: (1) a special affinity tag is used to react with cysteine residues in a protein; (2) labeled peptides are obtained from the enzymatic digestion of labeled proteins; and (3) labeled peptides are separated by LC or MDLC followed by MS analysis for relative quantitative measurements. ICAT is often used to measure the relative protein abundance between two samples, such as tissues from a healthy person and a patient. However, the analysis is usually long and expensive because of high costs of isotopic labeling reagents.

Isobaric tags for relative and absolute quantitation

The quantitation strategy for isobaric tags for relative and absolute quantitation (iTRAQ) labeling [33] of multiplexed sets of experiments is commonly based on a set of four isobaric reagents, each of which comprises three groups: reporter (mass from 114 to 117 Da), balance (mass from 28 to 31 Da), and reactive groups. After cell lysis, reduction/alkylation, and protein digestion, the peptides in four states are separately labeled on *N*-terminal and internal lysine residues by the reactive group of iTRAQ. Each labeled peptide appears at the same mass in a MS scan. During the fragmentation in the MS, the label dissociates and releases the reporter group as a singly charged ion of masses 114, 115, 116, or 117 Da, respectively. Relative intensity of these peaks indicates the contribution of each sample to total peptide intensity and can provide the information on the relative abundance. The labeled peptides are then combined and analyzed by MDLC and MS. The multiplexed protein quantitation capability of the iTRAQ strategy permits simultaneous comparison of samples of different cell states and disease specimens. This advantage can be applied to a comparative study on the temporal and/or spatial distribution of proteins in cells. iTRAQ can be used to simultaneously label and analyze up to four different samples. Absolute quantitative measurement of a specific protein can also be achieved by iTRAQ. This involves comparing peptides from a target protein to a known amount of labeled standard peptide added to the sample.

Stable isotope labeling with amino acids in cell culture

Stable isotope labeling with amino acids in cell culture (SILAC) was developed by Ong et al. [34] to detect proteomic differences between two cell samples. One of the cultured cell populations is fed with normal amino acids. In contrast, second cell population is fed with amino acids labeled with stable (nonradioactive) isotopes. For example, the medium can contain arginine labeled with six ^{13}C instead of the normal ^{12}C. During the cell growing period, they incorporate arginine into all of their proteins. Therefore, all heavy arginine-containing peptides are heavier than their normal counterparts by 6 Da. Pairs of chemically identical peptides of different stable isotope composition can be clearly distinguished because of their mass difference. The ratio of peak intensities for such peptide pairs can accurately reflect the population ratio for the two proteins. SILAC is becoming a powerful tool to study cell signaling [35—37].

H_2O^{18} labeling

Both ICAT and iTRAQ involve specific reagents for labeling. Another simple and inexpensive approach is by H_2O^{18} labeling during the process of trypsin digestion. One or two O^{18} can be incorporated into the carboxyl group of peptides. Mass peaks with M plus 2 and 4 can be expected [38,39]. This approach provides a very simple method for quantitative

measurement. However, analysis confusion may arise because of the interference peaks by other peptides in the digested buffer solutions.

Analysis and quantitation of 1D- or 2D-gel protein profiles

Before the development of PMF, proteomic studies were usually pursued by 1-DE or 2-DE. The visible bands or spots in 1D or 2D gel are based on isotope radioactivity labeling or staining with silver stain or Coomassie brilliant blue dye methods [24]. A 2-DE gel is difficult to do automatic data analysis when a high number of 2D gel pictures need to be analyzed. Nevertheless, it is still broadly used in most biological laboratories when complex protein mixtures of a specific tissue sample of interest need to be analyzed based on the global distribution of protein expression profile at a particular developmental stage. Each visible band or spot may represent one or a few proteins. To identify the proteins in the specific band or spot, the gel can be cut out for enzymatic digestion followed by PMF spectrometry. When two 2D gel pictures are compared to single out the clear differences of corresponding proteins, 2-DE is a convenient tool to find out different proteins with characteristic PMF used for protein ID (Fig. 3). Analysis of 2D gels in its simplest form can be carried out by superimposing one photographic image over another. However, better and more accurate results can usually be obtained by computer analysis of the gels.

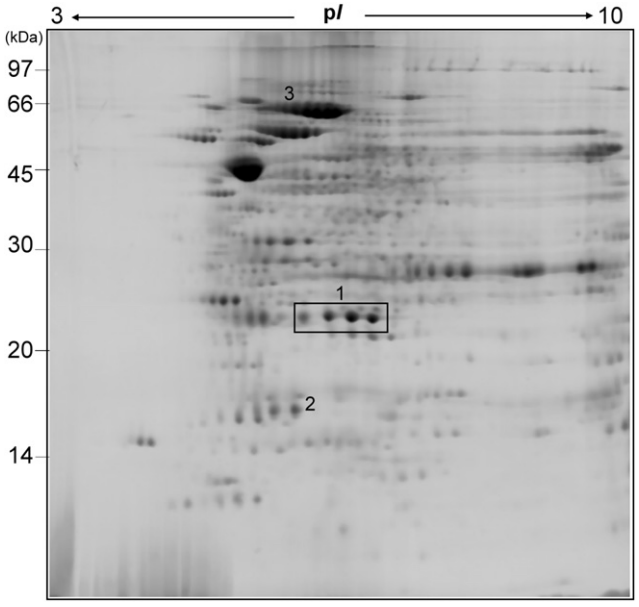

Figure 3. Protein expression profile in 2D gel of *H. pylori* cultured in BHI culture media. 250 μg of total proteins from lysates of *H. pylori* cells were loaded on IPG gel strips (pH 3—10, 13 cm) for the first-dimensional separation (IEF) followed by SDS-PAGE on the second dimension. Protein spots marked by No. 1—3 on the map were further identified by nano LC-MS/MS to be alkylhydroperoxide reductase (HP-AhpC), nonheme-iron ferritin (HP-ferritin), and urease B. The result is representative of three independent experiments. (Courtesy of Japanese Biochemical Society; [80]). BHI = brain heart infusion; *H. pylori* = *Heliobacter pylori*; IEF = isoelectric focusing; IPG = immobilized pH gradient; LC-MS = liquid chromatography coupled tandem mass spectrometry; SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Reproducible gels from which data can be collected on several hundred and perhaps over a thousand proteins from a single gel of one sample can now be analyzed automatically by scanning (gel scanner) and computer analysis with various versions of computer algorithms for data collection and analysis [40]. The system must be able to compare and match up patterns from several gels to allow accurate identification of spots for quantitative analysis. With the recent advent of powerful personal computers and the associated analysis softwares, it has become increasingly obvious that 2D protein gels can now be satisfactorily and reproducibly analyzed if they are digitized and the features of protein data displayed on the gels abstracted using an automated system. A 1D SDS-PAGE (Fig. 2A) is especially more straightforward and simpler than any previous method of protein analysis principally because it can be used to separate any proteins regardless of their inherent solubility in aqueous solution.

Although other physical methods can also give rise to the same information of molecular mass estimation afforded by SDS-PAGE, they lack the simplicity in the procedure and short time required for SDS-PAGE. Most noteworthy is that SDS-PAGE is a relatively simple and reproducible technique, which requires only micrograms of samples yet is capable of very high resolution as it can separate proteins differing in mobility by as little as 1% (a difference in molecular mass of 1 kDa for a 100 kDa protein), inferior only to the above-mentioned MS methodology. It should be kept in mind that in great contrast to current state-of-the-art MS, the molecular mass and quantity estimation by 1-DE or 2-DE is basically a relative method, which requires that molecular mass marker proteins be included with each gel run and a standard curve drawn for that particular gel under the specified conditions.

Comments on quantitative proteomics

All the above methods including ICAT, iTRAQ, SILAC, H_2O^{18}, and 2D gel are in general designed for relative quantitative proteomic analysis and comparison of selected proteins among different pair-wise samples under some specified conditions. In contrast, methods for absolute quantitative protein determination are more desirable than the relative methods from the quantitative aspects of proteomic approaches. Proteomics based on absolute quantitative analysis is aimed to measure the absolute level of protein biomarkers using a characteristic peptide unique to the studied protein. Absolute quantification and stable isotope standards and capture by antipeptide antibodies (SISCAPA) are two reported approaches for absolute quantitation [41—44]. For absolute quantification, peptides generated from specific enzymatic digestions are synthesized with heavy isotopes. A known quantity of the synthesized peptides with known molecular masses was added to proteomic samples as internal standards for intensity comparison to determine the quantities of the studied proteins. For SISCAPA, antipeptide antibodies immobilized on novel nanoaffinity columns are used to concentrate and enrich specific peptides along with stable isotope-labeled internal standard of the same sequences. On elution from the antipeptide antibody affinity column, ESI-MS is then used to determine the quantities of peptides by comparison of the signals from the peptides with those of the corresponding stable isotope-labeled internal standards [45].

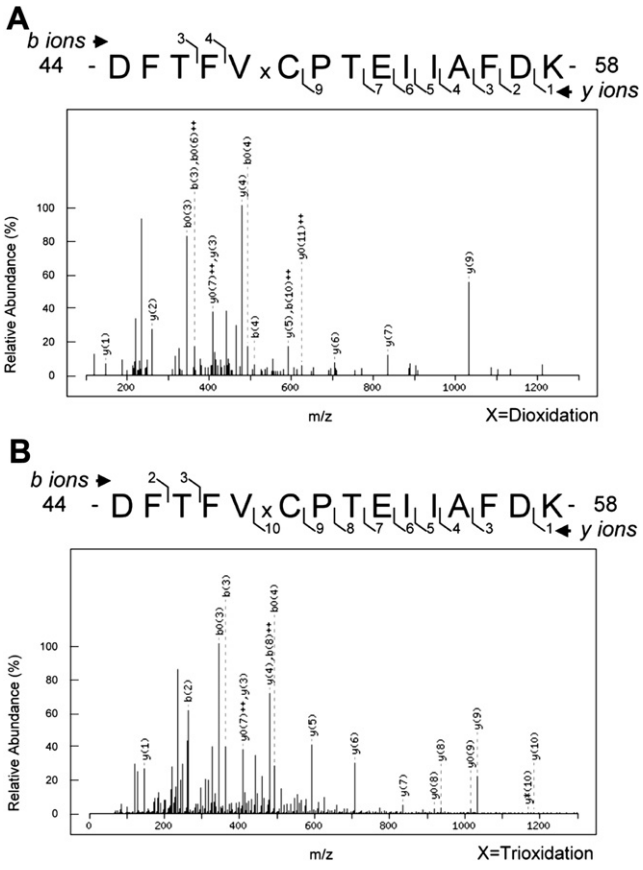

Figure 4. Identification of *in vivo* oxidized products of AhpC from *H. pylori* by LC-MS/MS. (A) The spectra of the digested peptides from oxidized HP-AhpC as analyzed and plotted by MASCOT database search program. The MS/MS fragmentation pattern shows the presence of the Cys49-containing peptide DFTFVCPTEIIAFDK, in which Cys49-SH at the catalytic site has been converted to Cys49-SO2H. (B) The spectra of the digested peptides from oxidized HP-AhpC as analyzed and plotted by MASCOT program. The MS/MS fragmentation pattern shows the presence of the Cys49-containing peptide DFTFVCPTEIIAFDK, in which Cys49-SH at the catalytic site has been converted to Cys49-SO3H. (Courtesy of Japanese Biochemical Society; [79]). *H. pylori* = *Heliobacter pylori*; LC-MS = liquid chromatography coupled tandem mass spectrometry.

Multiple reaction monitoring coupled tandem MS quantitative proteomics

Quantitative targeted proteomics focusing on multiple reaction monitoring-MS (MRM-MS) methodologies has taken front stage in the proteomics research recently, especially being frequently applied to the verification of global proteomics data, the discovery of lower abundance proteins, protein PTMs, and biomarker discovery [46]. As an older methodology used for small molecule analysis, MRM-MS has been adapted as the next methodological advance to address previously challenging issues in global proteomics experimentation, namely dynamic range, identification of PTMs, and sensitivity/selectivity of measurements. These developments have shown that targeted MRM analysis on nonredundant peptides of conventional MS/MS data of potential biomarkers can provide more exact quantification of homologous proteins. The methodology has facilitated the transition from routine biomarker-discovery proteomics to quantitative targeted-proteomics analysis, further allowing us to identify and verify more low-abundance potential biomarkers.

MRM is the extended version of Selected Reaction Monitoring (SRM), which arose as a technology to complement the discovery capabilities of shotgun proteomic strategies by its unique capability for reliable quantification of biomolecules (or termed analytes) present at low abundance in complex mixtures [47]. SRM exploits the unique capabilities of triple quadrupole MS for quantitative analysis, which is ideally suited to address a major proteomic challenge in systems biology for the accurate quantification of some specific sets of proteins spanning the whole range of cellular proteomes from different samples of evolutionarily related organisms.

In SRM, the first and the third quadrupoles act as filters to specifically select predefined m/z values corresponding to the peptide ion and a specific fragment ion of the peptide, whereas the second quadrupole serves as collision cell. Several such transitions from precursor peptide to their fragment ion pairs are monitored over time, yielding a set of chromatographic traces with the retention time and signal intensity for a specific transition as coordinates. The two levels of mass selection by two quadrupoles with narrow mass windows result in a high selectivity, as coeluting background ions are filtered out very effectively.

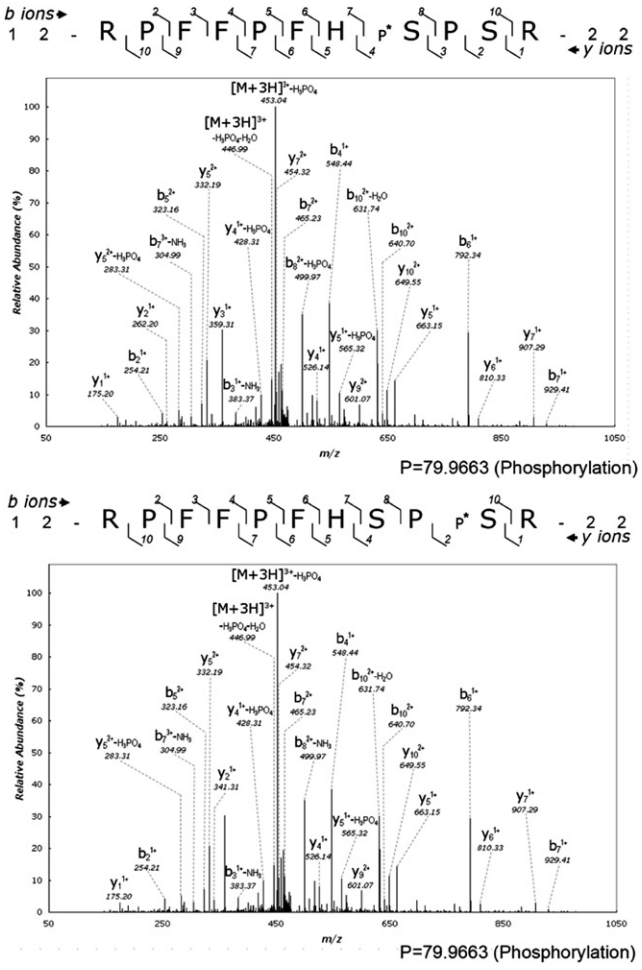

Figure 5. The representative tandem mass spectra of the phosphorylated peptide ^{12}RPFFPFHS*PS*R^{22}. (A) MS/MS spectrum of the peptide phosphorylated at Ser-19. (B) MS/MS spectrum of the peptide phosphorylated at Ser-21. The purified phosphopeptides samples less than 1 μg each from IMAC were first injected into a 2 cm × 180 μm capillary trap column followed by LC-MS/MS and spectra collection. Based on the tandem mass spectra of the modified peptide ^{12}RPFFPFHS*PS*R^{22} as compared with the original peptide, it can be deduced that both Ser-19 and Ser-21 are phosphorylated. The location of the peptide fragment within the protein is shown by the residue numbers 12 and 22 for the *N*- and *C*-terminus of the phosphorylated peptide sequence. Identified b- and y-ion fragment series are marked by the numbers above and under the peptide sequence, respectively. The putative site of phosphorylation is indicated by the P* in front of serine residues. The mass signals were amplified by fivefold except the ion with the highest intensity. (Courtesy of Molecular Vision Press; [63]). IMAC = immobilized metal affinity chromatography; LC-MS = liquid chromatography coupled tandem mass spectrometry.

Unlike other MS-based proteomic techniques, no full mass spectra are recorded in triple quadrupole-based SRM analysis. The nonscanning nature of this mode of operation translates into an increased sensitivity by one or two orders of magnitude compared with conventional "full scan" techniques. In addition, it results in a linear response over a wide dynamic range up to five orders of magnitude. This enables the detection of low-abundance proteins in highly complex mixtures, which is crucial for systematic quantitative studies of biomolecular samples.

It is also important to note that SRM and MRM have been successfully applied to a variety of biological applications, including robust quantitative proteomic analysis of cellular signaling networks [48], quantification of DNA adducts purified from tissue [49], and detection of doping substances in human urine and plasma [50—53]. To date, the use of SRM and MRM in proteomics is less widespread, although MRM has been used to quantify protein expression [54], to find protein biomarkers for disease severity in rheumatoid arthritis [55], and to detect and quantify protein phosphorylation profiles [48,56—58].

Phosphoproteomics based on immobilized metal affinity chromatography and LC-MS/MS

PTM of genome-encoded polypeptides is prevalent (about 10%—50% of proteins are phosphorylated) in various biological systems, which commonly includes acetylation, methylation, deamidation, glycosylation, phosphorylation, and various proteolytic truncations [59—62]. Up to now, most proteomics studies on the analysis of PTM are limited to

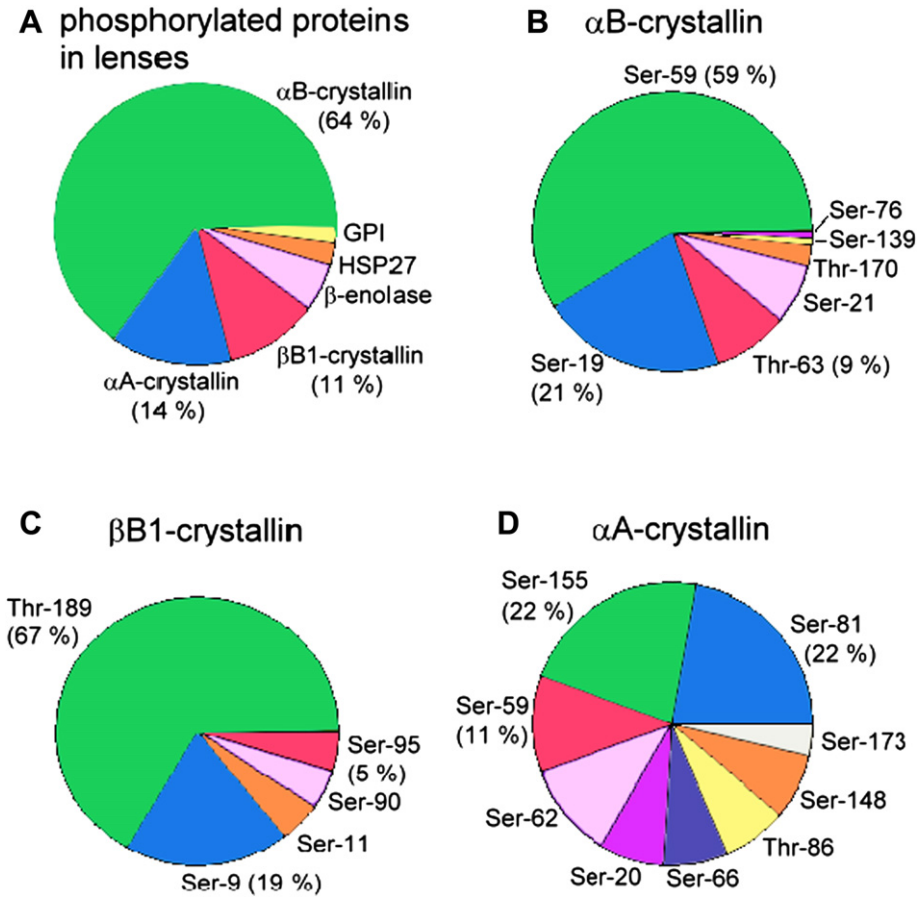

Figure 6. The percent distribution of phosphorylated sites identified by gel-free IMAC enriched phosphopeptide and LC-MS/MS analysis. (A) Proportion of the proteins with phosphorylation in total lens extract. (B) Distribution of *in vivo* phosphorylation sites in αB-crystallin. (C) Distribution of *in vivo* phosphorylation sites in βB1-crystallin. (D) Distribution of *in vivo* phosphorylation sites in αA-crystallin. The three most abundantly phosphorylated proteins (%) in the lens are shown under the identified phosphoproteins in A—D. It is noted that phosphorylated sites of αA-crystallin are more evenly distributed along the protein molecule than αB- and βB1-crystallins, which show the predominant phosphorylation sites at residues 59 and 189 in αB- and βB1-crystallins, respectively. (Courtesy of Molecular Vision Press; [63]). IMAC = immobilized metal affinity chromatography; LC-MS = liquid chromatography coupled tandem mass spectrometry.

phosphorylation and glycosylation. Especially, it is well known that phosphorylation is most noteworthy for playing a major role in the regulation of various biosignaling pathways [61], which may include ion transport, enzyme activation or inhibition, protein degradation, cancer development, and aging. Phosphorylated proteomics has been used to quantify dynamic changes in phosphorylated proteins over time. Herein, we present our data on quantitative phosphoproteomics using shotgun proteomic approach coupled with immobilized metal affinity chromatography (IMAC) and LC-MS/MS (Figs. 4 and 5). Identification of protein phosphorylation and its exact locations in proteins or enzymes of interest are always considered as a preeminent and nontrivial task in the conventional mechanistic and functional study of various cellular proteins. Mainly attributable to the advent of emerging proteomics, the investigation of protein phosphorylation has recently become less tedious and more amendable to routine analysis [28—30]. The common strategy of most conventional proteomic approaches to the identification of phosphorylated proteins rests in the PMF of proteins under study, which can be used as an identification tag to search the corresponding identical or highly homologous sequence fragment patterns in protein sequence databanks. Such fingerprints usually come from the tandem mass spectra of peptides generated from proteolytic digestion of proteins of interest.

As mentioned above, the serious drawback of low sensitivity and underrepresentation for some special classes of proteins, such as the extremely basic or acidic groups of proteins and membrane proteins, by using gel-based 1D or 2D gel proteomics [24—27] necessitated the development of more sensitive labeling methods, such as stable isotopic labeling [34—37], in conjunction with multidimensional LC-MS/MS analysis. Methodologies to date still do not exist for conducting a routine and reliable high-throughput analysis of proteome-wide changes in the phosphorylation of proteins. In our pilot study, phosphorylated and nonphosphorylated lens proteins from porcine eye lenses were first identified by gel-based 2D gel protein fractionation and separately by gel-free enrichment of phosphopeptides from trypsin-digested protein mixture on IMAC followed by LC-MS/MS. Based on our results of the

comparison and evaluation of two different protocols of proteomic approaches, we conclude that gel-free IMAC phosphopeptide enrichment, coupled with LC-MS/MS analysis, is now capable of identification of phosphorylated sites from the whole lens extract, effectively circumventing the need for prior protein separation by 2-DE. Our results clearly showed that two subunits of α-crystallin, αA-crystallin, and αB-crystallin (Fig. 2B), as well as other lens crystallins and noncrystallin cellular proteins, such as β-enolase, heat shock protein β-1 (HSP27), and glucose-6-phosphate isomerase, were found to be phosphorylated *in vivo* at specific sites. Moreover, αA- and αB-crystallins were found to be the most abundantly phosphorylated proteins in porcine lenses, being extensively phosphorylated on serine or threonine but not on tyrosine residues. Technically, the gel-free IMAC method facilitates direct site-specific identification of phosphorylation residues in lens proteins. Moreover, the improved strategy using gel-free phosphoproteomics analysis affords a more effective and simplistic method for the determination of *in vivo* phosphorylation sites than the conventional 2-DE preseparation of protein mixture. Therefore, the adaptation of quantitative shotgun proteomics in protein phosphorylation study (Fig. 6, [63]) proves to be very useful and form a firm basis for the future comprehensive analysis of other PTM in our lens protein system in terms of aging and various diseased states.

Conclusion

In the postgenomic era, the development of technologies for systematic, qualitative, and especially quantitative proteome analysis of biological samples from cells or tissues of biological systems especially humans has become an important focus of biomedical research. MS-based analytical methodology is now widely used for screening for inborn errors of metabolism [64,65], study of different types of cancer [66—68], viral or bacterial infections [69,70], cardiomyopathic heart disease [71], neurodegenerative disorders, such as Alzheimer's [72] or Huntington's [73] diseases, and even some applications in forensic medicine [74,75]. In our study of biomarkers related to *Helicobacter pylori*-associated gastroduodenal diseases by a proteomic approach (Figs. 3 and 4), we have identified several crucial pathogenesis-related proteins that have diagnostic and prognostic potential. Among them, antibodies to GroEs and AhpC of *H. pylori* [76,77] could probably be used for identification of patients who are at high risk of disease complications after *H. pylori* infection. Indeed, proteomics technology has been proved to be a tremendously powerful tool for simultaneously determining the presence of proteins and protein variation on a large scale in biological samples collected from *H. pylori*-infected patients. This newly developed and fast-evolving methodological advance has provided unprecedented opportunity to survey a cell's translational landscape in a global and comprehensive way to allow in-depth analyses of host and pathogen interaction. Using this MS-based proteomics platform and taking advantage of complete sequences for both the *H. pylori* and the human genomes in various databases, some promising results and important information have been derived [80,79,78,81]. On the other hand, the salient application of improved shotgun quantitative phosphoproteomics led to the effective and facile determination of previously unreported *in vivo* phosphorylation sites (Fig. 6) in our study of whole tissue extract from various animal eye lenses. The methodology is certainly to play a major role to elucidate the biological significance of phosphorylation in relation to various biosignaling pathways [82,83].

Regarding the recent development of quantitative clinical proteomics with its main focus on "biomarker discovery," more than 20,000 protein or peptide potential biomarkers have been revealed for cancer and other diseases in the literature. However, only a few candidates have been verified and validated by the United States Food and Drug Administration, in fact only seven protein biomarkers being approved between 2003 and 2008 [84,85]. The reason for this low success rate for verified and validated biomarkers is that only a small number of the reported candidate biomarkers have passed verification stage [86—88], and even fewer have been validated [89,90], which generally requires measuring the proposed biomarker in even larger cohorts of patients. On the practical side, to date, a complete proteomic analysis of one sample with the best MS instrumentation still needs at least one whole day. For a biomarker discovery aiming for a particular disease, it may need less than 10 samples (i.e. numbers of normal and patients), whereas biomarker verification can require 100—1,000 samples and biomarker validation requires even larger numbers of samples (thousands to tens of thousands) to be certain that the target molecule is a true biomarker of the disease and the "false-positive" rate of the developed assay can be estimated [88]. Therefore, it will take at least one whole year to accomplish such a large-scale screening and survey. Taking into a further account of analysis for phosphoproteomics, glycoproteomics, metabolomics, and so forth, it should take even longer to finish a comprehensive study for a validated biomarker. It is clear that innovative new technologies need to be developed to overcome this time limitation.

Future perspectives

Although we have witnessed tremendous progress in proteomics for the past decade (since the year of 2000), several major concerns and challenges need to be addressed to make a further transition to health and disease-related clinical proteomics in the future. At present, the bottlenecked obstacles are (1) the lack of established and routine MS-based analytical technology for PTM; (2) limited MS sensitivity, low dynamic ranges, and low resolution for biomolecules measured at high m/z ratios of greater than 100,000 for most MS instruments other than MALDI-TOF; and (3) undesirable long analysis time needed for clinical biomarker verification and validation. It is true that pre-MS analysis methodologies, such as electrophoresis and high-performance liquid chromatography are also time consuming. ESI-MS also takes more time than MALDI. An improved MALDI-TOF technology with the mass resolution reaching that of a Fourier-transform ion cyclotron or ion trap MS would be a better MS for a more satisfactory and high-throughput analysis in clinical proteomics. An automated, high-throughput, and multiplexed method using MRM coupled MS-based quantitative proteomics

(MS-MRM or MS/MS-MRM) has been proposed, which is very promising for clinical trials of protein/peptide biomarkers [91,92].

Finally, the ultimate goal for proteomic researchers is to be able to do single cell proteomics especially for cancer or stem cell research. Recently, single cell trapping and measurement of the mass of a single cell have been successfully demonstrated [93—95]. It should be feasible to break up the trapped cell and release small and large biomolecules for MS-based ionization and fragmentation, which may result in unraveling the detailed mechanistic pathways for the metabolic transition of macromolecules to small organic component molecules.

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