

BREAKTHROUGHS AND VIEWS

The SELDI-TOF MS Approach to Proteomics: Protein Profiling and Biomarker Identification

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The need for methods to identify disease biomarkers is underscored by the survival-rate of patients diagnosed at early stages of cancer progression. Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a novel approach to biomarker discovery that combines two powerful techniques: chromatography and mass spectrometry. One of the key features of SELDI-TOF MS is its ability to provide a rapid protein expression profile from a variety of biological and clinical samples. It has been used for biomarker identification as well as the study of protein-protein, and protein-DNA interaction. The versatility of SELDI-TOF MS has allowed its use in projects ranging from the identification of potential diagnostic markers for prostate, bladder, breast, and ovarian cancers and Alzheimer's disease, to the study of biomolecular interactions and the characterization of posttranslational modifications. In this minireview we discuss the application of SELDI-TOF MS to protein biomarker discovery and profiling.

The discovery, identification, and validation of proteins associated with a particular disease state is a difficult and laborious task and often requires hundreds, if not thousands, of samples to be analyzed. Presently, disease biomarker discovery is generally carried out using two dimensional polyacrylamide gel electrophoresis (2D-PAGE) to compare and identify differences in the protein expression patterns between diseased and normal samples. After 2D-PAGE fractionation and staining, the protein(s) of interest are removed, proteolytically or chemically digested and identified by mass spectrometry (MS). Although 2D-

PAGE separation provides excellent resolution, the need for protein staining and the subsequent sample handling limits the sensitivity of the overall approach. Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a novel approach, which was introduced by Hutchens and Yip (1). The ProteinChip Biology System uses SELDI-TOF MS to retain proteins on a solid-phase chromatographic surface that are subsequently ionized and detected by TOF MS. This versatile instrumentation is presently being used for projects ranging from the identification of disease biomarkers to the study of biomolecular interactions. The ProteinChip Biology System allows for protein profiling from a variety of complex biological materials such as serum, blood, plasma, intestinal fluid, urine, cell lysates, and cellular secretion products with limited sample preparation and for protein-protein and protein-DNA interactions (2–19). The system is most effective at profiling low molecular weight proteins (i.e., <20 kDa), providing a complementary visualization technique to 2D-PAGE. SELDI-TOF MS, however, is more sensitive, and requires smaller amounts of sample than 2-D PAGE. Protein profiles have been generated from as few as 25–50 cells (5, 6).

The ProteinChip platform has been used to discover biomarkers for diseases such as cancer (2–8), neurological disorders (9) and pathogenic organisms. It has proven to be useful in the discovery of potential diagnostic markers for prostate (3–7), bladder (8), breast (9) and ovarian (10) cancers, Alzheimer's disease (11), and profiling of urinary proteins to assess renal function (12). ProteinChip Arrays have also been used to characterize protein-protein interactions (13, 14), phosphorylated and glycosylated proteins (15, 16), and transcription factors (17). Sato *et al.* (18) used SELDI-TOF MS to profile low molecular weight peptides and proteins secreted by different cancer cell lines grown in

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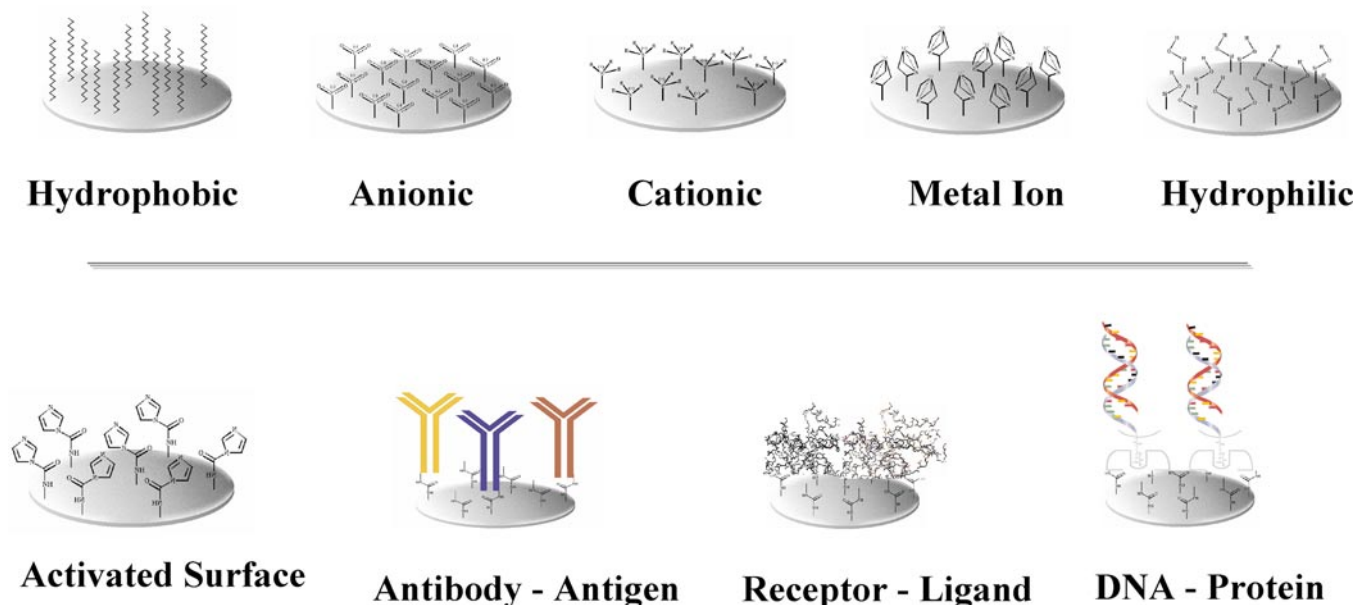


FIG. 1. The variety of ProteinChip arrays available for sample preparation. The arrays are comprised of either 8 or 16 spots comprised of a specific chromatographic surface. The upper arrays represent chemically modified chromatographic surfaces while the bottom arrays are biochemically modified surfaces. Chemically modified surfaces are used to retain a group of proteins based on a specific physical property such as hydrophobicity, charge, etc. Biochemically modified surfaces are typically used to isolate a specific protein or functional class of proteins.

serum free medium. The chromatographic surfaces that make up the various ProteinChip Arrays are uniquely designed to retain proteins from a complex sample mixture according to specific properties such as hydrophobicity, charge, etc. The molecular weights of the retained proteins can then be measured by TOF MS. This mini review will discuss the application, advantages and limitations of the SELDI-TOF MS approach to protein profiling and protein biomarker detection and identification.

INSTRUMENTATION

The SELDI-TOF MS technology consists of three major components: the ProteinChip Array, the reader, and the software. The ProteinChip Array is a 10-mm-wide \times 80-mm-long chip having eight or sixteen 2-mm spots comprised of a specific chromatographic surface (Fig. 1). Each surface is designed to select proteins from crude extracts according to general or specific protein properties. Each spot contains either a chemically (anionic, cationic, hydrophobic, hydrophilic, metal, etc.) or biochemically (antibody, receptor, DNA, enzyme, etc.) treated surface. Typically, surfaces treated with a biochemical agent, such as an antibody or other type of affinity reagent, are designed to interact specifically with a single target protein, while chemically treated surfaces retain whole classes of proteins. The biochemical surfaces are custom-made by the user on an open preactivated platform surfaces to which the users attach their own bait molecule. Any crude extract or sample can then be applied to the surface to promote

interactions with the bait molecule. The biochemical surfaces exploit specific molecular recognition mechanisms of antibody-antigen, enzyme-substrate, receptor-ligand and protein-DNA interactions to selectively capture target proteins from complex biological specimen.

The procedure for detecting protein biomarkers is very simple. A few microliters of the sample are dispensed onto the ProteinChip surface under specific binding conditions that determines which proteins will be retained by the surface. Protein specificity is achieved through the application of a series of washes with an appropriate solvent or buffer designed to elute unbound proteins and interfering substances, such as salts, detergents, lipids, etc., while retaining the proteins of interest. After being allowed to dry, an energy absorbing molecule (EAM) solution is added, and the array is inserted into the ProteinChip Reader to measure the molecular weights of the bound proteins.

The ProteinChip Reader is a laser desorption ionization (LDI)-TOF MS instrument equipped with a pulsed UV nitrogen laser source (Fig. 2). Upon laser activation, the sample becomes irradiated and the desorption/ionization proceeds to liberate gaseous ions from the ProteinChip Arrays. These gaseous ions enter the TOF MS region of the instrument, which measures the mass-to-charge ratio (m/z) of each protein, based on its velocity through an ion chamber. Time lag focusing is used to increase the mass accuracy of the final output. Signal processing is accomplished by high-speed analog-to-digital converter, which is linked to a personal computer. Detected proteins are displayed as a series of peaks.

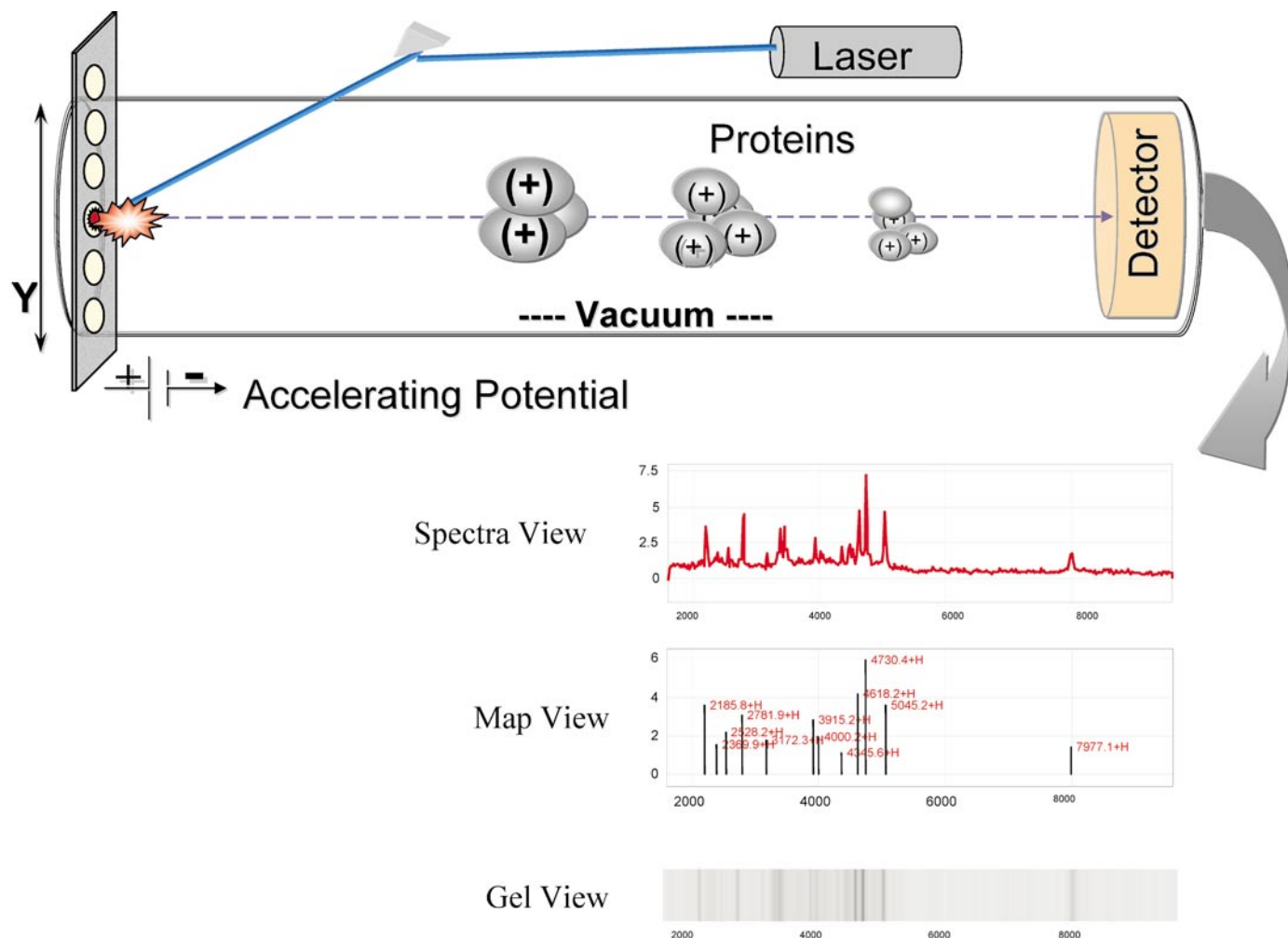


FIG. 2. Schematic diagram of the SELDI CIPHERGEN mass spectrometer. After sample preparation the ProteinChip arrays are analyzed by a laser desorption ionization (LDI) time-of-flight mass spectrometer (TOF MS). The TOF MS measures the molecular weights of the various proteins that are retained on the array. For comparison purposes, the software associated with the SELDI CIPHERGEN instrument is capable of displaying the resultant data as either a spectra, map, or gel view.

One of the primary uses of SELDI-TOF MS is to identify differences in the protein expression profiles of two or more distinct samples. The samples being analyzed are often quite complex, particularly in the field of biomarker discovery where protein profiles of clinical samples are measured. The output generated from the TOF MS analysis of such samples is a trace showing the relative abundance versus the molecular weights of the detected proteins (Fig. 3). To identify differences in protein abundances between two samples, software has been developed that converts the peak trace into a simulated one-dimensional gel electrophoresis display (Fig. 2). The software compares these displays to identify bands unique to one sample or proteins that show a significantly greater relative abundance in one of the samples.

The end result of a SELDI-TOF MS analysis is a list of the molecular weights of proteins whose relative abundance differs significantly between two or more

samples. The next step is the actual identification of the differentially expressed protein(s). Identification most often requires the development of a strategy to purify the differentially expressed protein(s). The purification strategy can be based on any type of chromatography; however, the type of ProteinChip Array that the protein(s) of interest bind to provides a useful starting point in the experimental design. The goal is to purify the species that matches the molecular weight of the protein identified as being differentially expressed in the SELDI-TOF MS analysis. Once purified, standard MS-based methods, such as peptide mapping or tandem MS, can be used to identify the protein. A new ion source has been developed that allows the ProteinChip Arrays to be analyzed using a Q-STAR hybrid triple quadrupole/TOF MS. With this new source, proteins can be tryptically digested directly on the arrays and the resultant fragments identified by tandem MS using the Q-STAR MS.

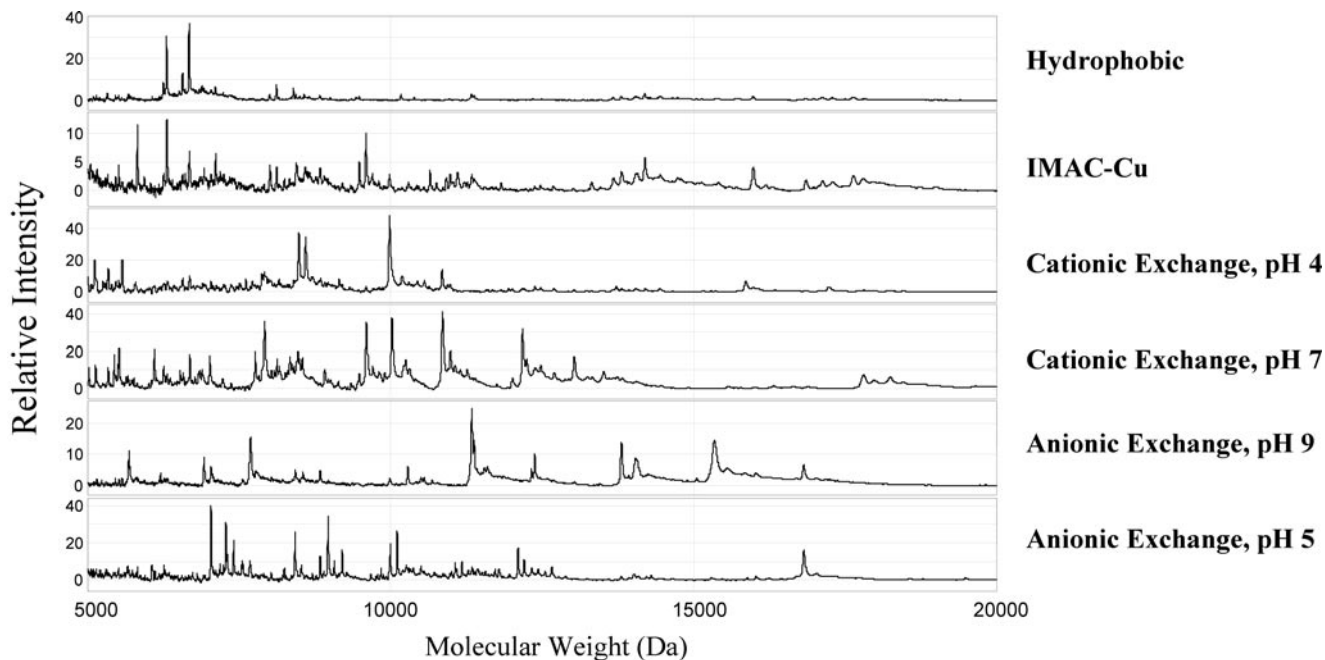


FIG. 3. Protein profile of a cell lysate on different ProteinChip surfaces. As shown above for a selection of ProteinChips, the individual surfaces retain different groups of proteins depending on their physiochemical properties. In addition, as shown for the cation and anion exchange surfaces, the proteins retained are also dependent on the pH of the sample.

APPLICATIONS

The versatility of the SELDI-TOF MS approach to protein analysis has been demonstrated by a number of published applications (2–19). The unlimited type of samples and the unique surface chemistries of the arrays allow the researcher to exploit chemical and biochemical characteristics of protein families, to capture and retain proteins on the surface, and to analyze the captured proteins by TOF MS or MS/MS directly on the array.

As a discovery tool, SELDI-TOF MS can be used to detect protein expression patterns from defined population sets (5, 10). The procedure is very simple. Blood or tissue specimen are taken from the patients and distributed onto different ProteinChip Arrays with different chemistries and a protein profile is achieved. By surveying different population sets (diseased vs normal, treated vs control, differentiated vs immature) through the interactions of the proteins with the ProteinChip Array surfaces, unique protein expression profiles can be produced similar to those shown in Fig. 3. A specific pattern can be distinguished using statistical programs such as Biomarker Patterns Software (20). The results can be used to as a clinical diagnostic to delineate the stage of the disease and/or the utility of treatment. Pattern recognition studies do not depend on detecting low abundance proteins but depend on fluctuations of protein expression patterns whether they are highly abundant housekeeping proteins or key proteins in a specific pathway.

Using the different array surfaces a complete picture of the disease state may be drawn and thus a better understanding of the disease and the required treatment. Petricoin and Liotta at the National Cancer Institute (5, 6, 10) have successfully used SELDI-TOF MS to generate protein fingerprint profiles of serum samples obtained from patients with breast, prostate and ovarian cancer. In their ovarian cancer study, they compared the protein profiles of serum samples from several patients with ovarian cancer to control patients. While no obvious, discrete peak was found to be diagnostic for ovarian cancer, an artificial intelligence program was able to decipher diagnostic “patterns” within the profiles. This same software program was then able to correctly diagnose serum samples as being taken from patients suffering from ovarian cancer or healthy patients. The exciting finding is that the SELDI Ciphergen-based analysis was able to diagnose patients at the earliest stage of cancer. Wright *et al.* (7, 19) has gathered data using SELDI-TOF MS that predicts prostate cancers with specificities and selectivities better than the currently accepted protein specific antigen (PSA) tests; for example they used ProteinChip immunoassay for the detection and quantification of prostate-specific membrane antigen and were able to use the results to discriminate between benign and malignant prostate disease (19). The ability to employ the results to diagnose disease, especially those cancers such as ovarian which presents at end-stage, and the severity of disease would have profound effects on the current clinical arena.

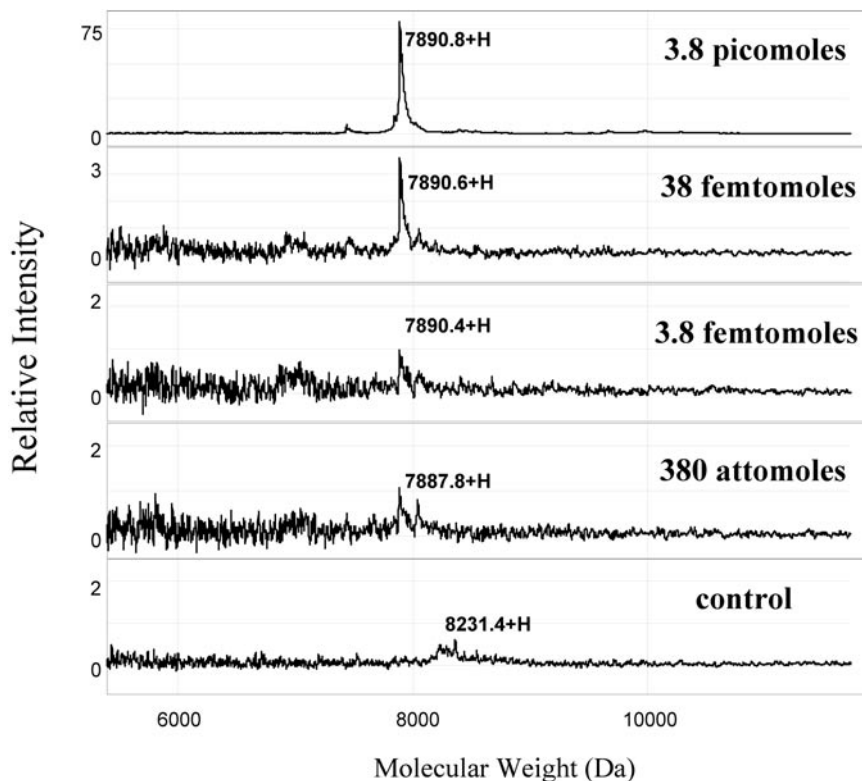


FIG. 4. Determination of limit of detection using antibody capture chip. In this analysis an antibody was covalently coupled to the ProteinChip surface and a solution containing various concentrations of its antigen were applied to the chip. While the detection limits are antibody-dependent, studies have demonstrated the ability to detect antigens in the attomole range.

The utility of SELDI-TOF MS is not limited to clinical proteomics but can be exploited as a companion platform in process proteomics. The micro-scale design of the array allows for small quantities to be examined and thus permits the testing of purification schemes prior to large-scale production. For example the strong anionic exchange (SAX2) array can be scaled to the Biosepra Q Ceramic HyperD anionic exchange resin. Thus binding and elution protocols may be investigated without losing large quantities of material.

Relevant applications, which may enhance basic research tools, include antibody capture studies. ProteinChip Array biological surfaces can be covalently modified with bait molecules, such as antibodies or recombinant proteins, to examine proteins that have an affinity to the bait. Antibody capture studies permit the examination of multiple isoforms when using the ProteinChip platform. If the antibody being used as the bait molecule recognizes an epitope in different isoforms of the protein, then these proteins can be separated by their molecular mass and will be detected. This direct sampling is a unique advantage over the more traditional ELISA method in which the resultant signal is a weighted average of all of the bound species. Detection limits are dependent on the antibody used but have been demonstrated to be at the attomole level (Fig. 4). Standard quantitation curves have been

shown to be linear over 2 to 3 orders of magnitude. The detection and identification of post-translation modifications can also be effectively accomplished using SELDI-TOF MS. SELDI-TOF MS is particularly effective at identifying glycosylated and phosphorylated proteins. Glycosylated proteins generally give a broad peak when analyzed by SELDI-TOF MS. The glycoproteins can be deglycosylated while still attached to the ProteinChip Array and then re-analyzed. The molecular weight of the sugar group is then determined by comparing the TOF MS spectra of the wild-type and deglycosylated forms of the glycoprotein (16). Another important application includes the use of SELDI-TOF MS to study the phosphorylation states of proteins involved in signal transduction pathways. A modification of 80 Da (phosphate group) can be easily detected on a peptide or a small protein. Kinase activity assays can be performed directly on the ProteinChip Array by covalently coupling a recombinant kinase to the activated array surface. In this method, the recombinant protein is bound to the biological array surface and a substrate peptide and ATP is added to the reaction buffer. After an incubation period, the phosphorylation state of the peptide is detected by an 80 Da increase in the peptide's molecular weight (15). The major advantage of this technique is that it does not require the use

of radioactive isotopes and a single assay can be sampled at many different time points.

In addition to the current applications, several investigators are using ProteinChip Arrays as an alternative to gel shift assays (17). Using the biological surface, streptavidin can be covalently coupled to the surface and biotinylated DNA or RNA can be bound to make a nucleic acid affinity surface. Nuclear extracts can then be examined for possible associations with specific consensus sequences of the DNA or RNA. Proteins bound to the nucleic acid can then be detected by TOF MS. Furthermore, any protein associating with the consensus sequence may be digested, and subsequently identified by MS/MS.

CONCLUSION

Disease biomarker discovery using SELDI-TOF MS is a novel approach that combines chromatography and MS. This novel approach to proteomics has been used for protein biomarker detection and identification and for the study of biomolecular interactions and post-translational modifications. The ProteinChip System allows protein profiling from a variety of biological materials with minimal sample preparation. It has been used for discovering proteins with potential application as diagnostic markers for detection of prostate, bladder, breast and ovarian cancers as well as Alzheimer's disease. ProteinChip Arrays have also been used for phosphorylation site mapping and glycoproteins characterization directly on the array. The high throughput ability of the SELDI-TOF MS system allows hundreds of samples to be screened for disease biomarker identification in a relatively short time period, providing investigators the opportunity to compare patient-to-patient variability.

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