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Protein Microarrays and Their Applications

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Abstract In recent years, the importance of proteomic works, such as protein expression, detection and identification, has grown in the fields of proteomic and diagnostic research. This is because complete genome sequences of humans, and other organisms, progress as cellular processing and controlling are performed by proteins as well as DNA or RNA. However, conventional protein analyses are time-consuming; therefore, high throughput protein analysis methods, which allow fast, direct and quantitative detection, are needed. These are so-called protein microarrays or protein chips, which have been developed to fulfill the need for high-throughput protein analyses. Although protein arrays are still in their infancy, technical development in immobilizing proteins in their native conformation on arrays, and the development of more sensitive detection methods, will facilitate the rapid deployment of protein arrays as high-throughput protein assay tools in proteomics and diagnostics. This review summarizes the basic technologies that are needed in the fabrication of protein arrays and their recent applications.

Keywords: protein microarray, immunoassay, diagnostics, proteomics

INTRODUCTION

In recent years, the complete sequencing of the human genome has allowed for the analysing gene expressions. For gene expression profiling, DNA arrays containing multicomponent microarrays have been reported [1,2], which provide much information about molecular assays, such as differential gene expression and DNA sequencing. Although this gene-level information plays an important role in applications, there are some limitations in that the DNA arrays can not provide protein level information, such as protein-DNA interaction, post-translational modifications and protein-protein interactions, which are very important in cell functions (Fig. 1). To get such information on a biological system, information on the state of proteins is required. Although the current technologies of protein analyses, such as electrophoresis with 2D-gel, have the ability to mine accurate data for protein analysis, they still have disadvantages with regard to high-throughput protein analysis. Therefore, it is necessary to develop protein-based high throughput analytical tools to increase the study protein functions. To tackle the problem of high-throughput analysis of proteins, much research has been taken to expand microarray technologies, which have been applied to DNA microarrays and protein-based microarrays. Many of these so-called protein microarrays, or protein chips, have been developed to fulfil the needs for high-throughput protein analysis, and applied to various fields, such as diagnostics, proteomics, biomarker discovery and drug screening. To develop the protein microarrays, like DNA microarray, biological, chemical and physical skills have to be combined to fabricate the protein microarrays for successful applications in these fields.

In this review, an overview of the present technologies for protein microarray fabrication and the application of protein arrays will be described (Fig. 2).

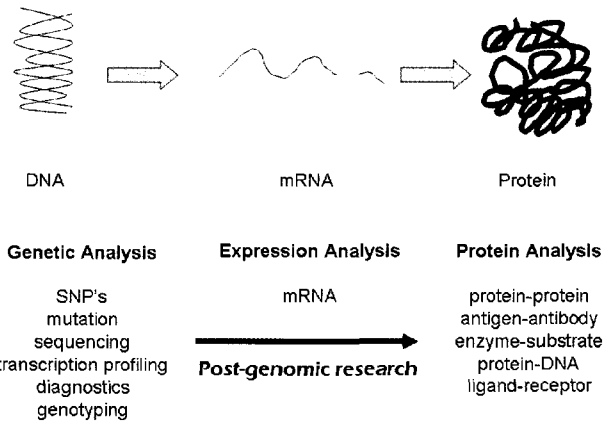

Fig. 1. Different physiological states for the cellular process. Protein microarrays could be applied to protein identification and quantification.

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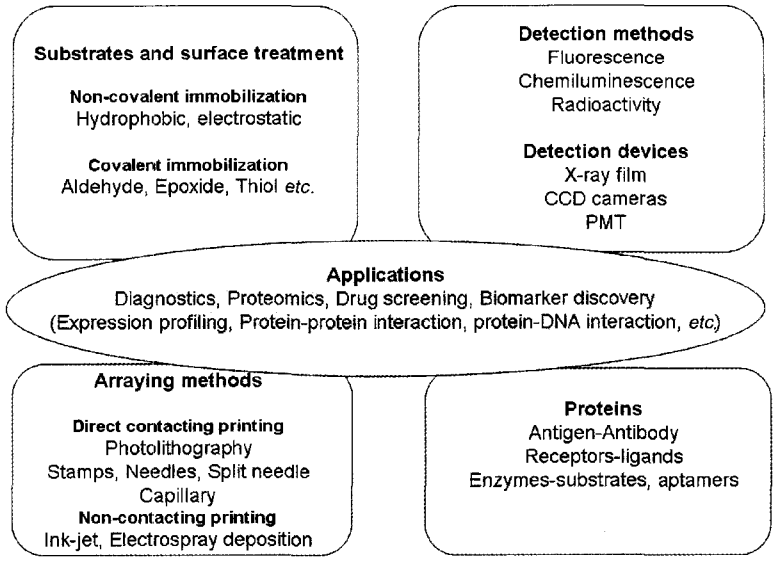

Fig. 2. Essential factors for the fabrication of a protein array and the possible applications.

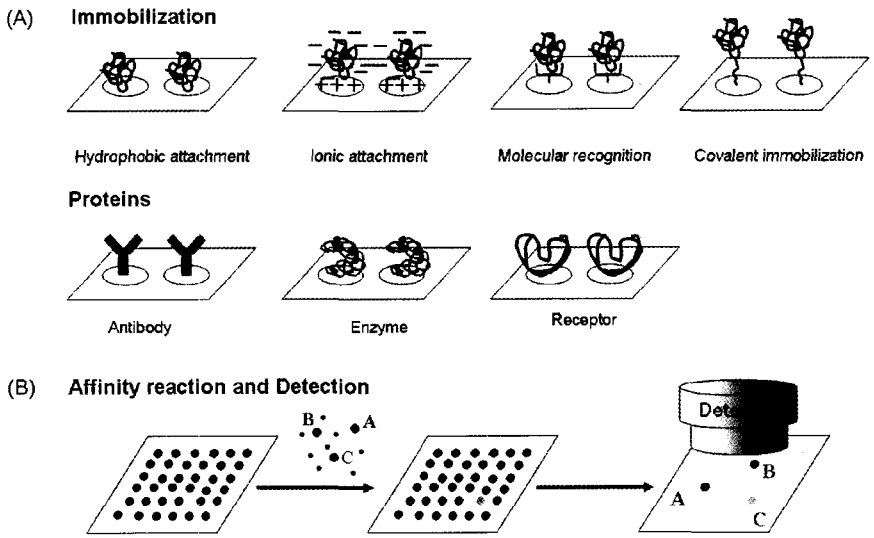

Fig. 3. (A) Diagram of various surfaces and classes of protein available for protein microarray assays. (B) Schematic representation of analysis using a protein microarray.

TECHNIQUES FOR THE FABRICATION OF PROTEIN ARRAYS

Substrates and Surface

Proteins can be immobilized using non-covalent or covalent attachments (Fig. 3). In the past, immobilization methods have relied on adsorption on to a surface, using hydrophobic interactions, due to its simplicity and cost-effectiveness. Proteins have usually been immobilized onto a polymer surface, such as polystyrene or nitrocellulose, using hydrophobic interactions, which is well established in enzyme linked immunosorbent assay (ELISA) or Western blotting [3,4]. Proteins can be also attached onto the intrinsically charged surface of a substrate or a surface modified with charged molecules, such as polylysine, using electrostatic interaction. The non-covalent attachment of a protein using hydrophobic interactions may cause the denaturation of the protein, such as unfolding, and may be weak for maintaining stability in an assay procedure due to the reversible nature of non-covalent attachments. Therefore, proteins are needed for immobilization with covalent bonding to enhance the stability of immobilized protein and the control of protein binding site availability. Using surface modification of substrates to the reactive moieties, such as aldehydes,

aminosilanes and esters, which can make covalent linkage with amine or carboxyl groups of a target protein, the efficiency of protein immobilization might be improved [5-8]. These surface techniques for protein immobilization have been established by many research groups in the development of processes for DNA microarrays, and products to meet the needs for the immobilization of biomolecules have been produced (*e.g.* Telechem international Inc., Zeptosens AG, Zyomix Inc., Versalinx™ technology, Prolinx Inc., and Bothell). Specific interactions, such as streptavidin-biotin or his-tag-nickel chelates, have also been used to immobilize proteins [9-12].

Although the covalent bonding of proteins to the surface-modified substrate can enhance the efficiency of immobilization, there are some drawbacks in that these methods might lead to denaturation of the proteins [13,14]. For example, several cross-linkers that immobilize proteins induce damage of the binding site. To overcome these problems, molecular adapter that can specifically bind the protein might be used. In the case of antibodies, Protein A, which is a 64 kDa bacterial surface protein extracted from the bacterium *Staphylococcus aureus*, has been used for oriented immobilization using the characteristic feature of binding to the Fc portion of IgG derived from several kinds of animals, which lead to a greater binding ability of the bound antibody because the Fab regions are free to the binding antigen [15-19]. Similarly, the carbohydrate moieties of the Fc region on an antibody can also be used for the oriented immobilization of an antibody, which allows the oriented antibody immobilization with good steric accessibility to the antigen binding site [20,21].

Arraying Methods

A number of studies have focused on the deposition of protein and other biomolecules in the fields of genomic, proteomic, clinical and pharmaceutical analyses. Methods that enable active protein arrays are rapidly developing, with many different types now appearing. Proteins have been deposited by direct contacting using photolithography [22-27]. Mooney *et al.* used an assembly of two-dimensional pattern of antibodies onto a silicon dioxide surface fabricated by UV photolithography, and a stamp microcontact printing method, where proteins can be immobilized by a stamping action with a micro-patterned elastomeric stamp [28,29]. Although these technologies have the advantage of well-defined protein micro patterning, there are crucial drawbacks that they need complicated process for multi-component printing with pattern in the fabrication of a multi-component microarray. Recently, the contact printing method has been developed, which employs tiny needles able to deliver nanoliter volumes directly onto the surface of substrates [30].

In addition to the direct contact printing methods, proteins can also be deposited using a non-contacting method that employs ink jetting [31-35] or the electrospray deposition (ESD) method [36]. The ink jetting method is well known to commercial printers. This device uses a piezo actuator that can dispense a droplet of the sample solution from the nozzle at the nanoliter level. The application of ink-jet devices to dispense several proteins, including glucose oxidase, horseradish peroxidase and laminin, has been performed. Recently, new commercial products for high throughput deposition, such as TopSpot™, have been presented. In the electrospray deposition (ESD) method, which was first used to fabricate thin radioactive sources in nuclear physics, solutions of a substance are electrosprayed, and charged particles deposited onto specified areas of a conductive substrate under the control of electrostatic forces. The electrospray deposition (ESD) method has recently become the centre of attention, as it allows spontaneous deposition of many identical dots, has a remarkable spatial resolution and overcomes limitations, like the low rate of deposition, low efficiency of substance transfer and cross-contamination. It has recently been reported that under proper conditions the ES-deposited proteins preserved their activity and were specifically recognized by antibodies [36].

Detection Methods

A number of different detection methods have been developed for bioassays and applied to the development of DNA microarrays that can also be adapted to detect protein targets. So far, the detection of proteins is usually performed by spectrophotometric methods, such as fluorescence or chemiluminescence, from probe molecules conjugated to capture molecules or target proteins. Several fluorescent probe molecules, such as Alexa Fluor series, R-phycoerythrin (RPE), Cy3 and Cy5 [37], have been evaluated for fluorescent bioassays. CCD-cameras or photon multiplier tubes (PMT) are used to detect fluorescent light, with high sensitivity, in many cases [38-40].

A high-sensitivity detection method for a microarray based on fluorescence-based wave guide technology has been reported. An array immunosensor for clinical analytes, using fluorescence-based wave guide method, has been developed by Rowe *et al* [41,42]. Captured targets were visualized with appropriate fluorescently labelled detection molecules. A dramatic increase in sensitivity and an adaptation to the microarray assay was achieved with the development of planar waveguide detection, as only surface bound fluorophores were excited [43,44].

Recently, the application of rolling circle DNA amplification (RCA) has also promised ultrasensitive detection for microarrays [45-47]. An oligonucleotide conjugated to a secondary antibody is used as the probe, and a DNA circle hybridizes to a complementary sequence in the antibody. The DNA tag is then amplified by RCA, and the amplified product labelled with the oligonucleotide conjugated fluorescent probe. The signal can be detected with high fluorescence sensitivity because it carries many fluorophores in the tag. The RCA showed a significant increase in sensitivity compared to conventional assays when applied to an assay using microarrays.

Using enzymes, such as a HRP, the chemiluminescent signals can be detected with CCD-cameras or photomultipliers, which have the advantages of better sensitivity and simpler optical setup than the fluorescent method.

The chemiluminescent signals can also be detected with x-ray films. Although this method provides insufficient resolution there are some merits in that the user can easily perform experiments using a simple device [36,48]. In addition to the conventional detection methods, a thermal lens microscope (TLM), useful for sensitive detection, has been developed [49]. As a labelling material for an antibody, colloidal gold particles are used, which have strong absorbance at ~500 nm. The TLM is composed of a microscope with two laser oscillation apparatus, excitation and probe beams. The signal from the gold particles attached to the secondary antibody is determined by thermal deflections of the probe laser beam. The TLM system has been applied to determine IgA, carcinoembryonic antigen (CEA) and other biomolecules [50-52]. In the case of CEA detection, which is a commonly used biomarker of colon cancer, the limit of detection was ~100 pg/mL, whereas generally, the detection limit of conventional immunoassays is 1 ng/mL. Although this method still has some drawbacks for commercialization, this system should be practicable due to its short analysis time and the ease of the procedures.

APPLICATIONS OF PROTEIN MICROARRAYS

Antibody-based assays with a microarray are the most common type of high-throughput bioassay, and the techniques involving the analysis of biomolecules have rapidly develop because of their specificity. Lee *et al.* describe a microassay fabricated using the ESD method, based on an ELISA format, for a high-throughput system [36]. The microarray fabricated with this method consists of over three hundred spots on a flat glass plate, which has proven to have microarray uniformity by AFM and FE-SEM. With six antigen-antibody sets, the sensitive and multiplex detection of arrayed antibodies has been shown to be feasible with fluorescent and chemiluminescent probes. Only minimal unspecific binding, with no cross-reactivity to non-specific proteins, has been observed. Other microarray-based approaches, which multiple IgG subclasses detection, can be performed, and have been demonstrated by Silzel *et al.* [53].

The protein microarrays can be applied to the detection of antibodies that react with self-antigens, which is crucial in the diagnosis and management of autoimmune diseases. The microarrays have been applied to the detection or screening of autoantibodies present in patients' sera [54]. Eighteen different autoantigens, commonly used as diagnostic marker for autoimmune diseases, such as systemic rheumatic disease, have been immobilized in microarray formants. After incubating patient sera, the arrays were detected with chemiluminescent reaction using a CCD camera. From less than 1 μL of a patient serum the autoantibody titers were determined with high accuracy. The microarray method can also be used to develop allergen profiling and IgE monitoring [55]. Barbara *et al.* described an *in vitro* test system for allergy diagnosis based on a microarray [56]. Allergen molecules immobilized on glass slides, activated with (3-glycidyloxypropyl)trimethoxysilane, are used to screen allergen-specific IgE against a multitude of allergens. The measurement of the microarray was performed automatically with using chemiluminescence intensities. With these microarrays, it is possible to distinguish between patients with and without elevated levels of allergen-specific IgE.

The determination of cytokines has been performed due to their importance in understanding immunological mechanisms. Microarray techniques can help the data mining of cytokine in patients for the correlation of multiple cytokine levels in disease progression. Assays using an antibody-based protein array for the detection of cytokines have been published by Huang *et al.* [48,57], where the capture antibodies cognate for cytokines are spotted onto a membrane in an array format. The reports demonstrate that several cytokines, including MCP-1 and TNFα, can be simultaneously detected using this approach at levels lower than 25 pg/mL. The detection of chemokines was performed using a microarray fabricated onto a hydrogel pad with fluorescent probes containing the same groups [58]. In addition to these reports, Barry *et al.* demonstrated that a total of 75 cytokines could be simultaneously measured with high specificity on a glass microarray, with signal amplification, by RCA [47].

Belov *et al.* describe a novel CD antibody microarray which enabled semi-quantitative identification of the expression of CD antigens on leukaemia and lymphoma cells from the peripheral blood of patients [59,60]. While many of the antibody arrays demonstrated have beeen designed to detect proteins, this array was designed to capture cells. The original antibody microarray consisted of 60 dots of CD antibodies against cognate surface antigens on human leukocytes. The microarray fabricated onto nitrocellulose was evaluated by numbering the cells captured by each CD antibody dot, using an optical scanner, with no staining or labelling. Extensive immunophenotypes of leukemias and lymphomas obtained with this microarray can be used for diagnosis in patients, and for basic research in cell biology.

Protein profiling can be facilitated with a microarray. Miller *et al.* developed a practical strategy for serum protein profiling using antibody microarrays, and applied the method to find potential biomarkers in prostate cancer serum. The micorarrays containing 184 unique antibodies were prepared, and serum samples labelled with Cy3 or Cy5 [61]. With a defined set of microarray measurements, five proteins; von Willebrand factor, IgM, α_1-antichymotrypsin, villin and IgG, were determined to be at significantly different levels between prostate cancer samples and the controls.

Protein arrays can also facilitate high-throughput proteomic studies. Microarrays of highly specific capture molecules may be used for direct protein identification, protein-protein interactions and DNA-protein interaction. MacBeath and Schneider developed microarrays that accommodated extremely low sample volumes, which enables the rapid, simultaneous processing of proteins [5]. Proteins, including protein G, p50, active domain of the human immunophilin FKBP12 and different kinase substrates, were covalently immobilized onto surface-modified

glass. The microarrays were incubated individually with cognate molecules labelled with fluorescent dyes. They demonstrated that the microarrays could be used in three applications, such as screening for protein-protein interaction, identifying the substrates of protein kinase and identifying the protein targets of small molecules. Zhu *et al.* demonstrated microarrays consisting of the proteins expressed from 5,800 open reading frames of yeast, which performed analyses for proteins interacting with proteins and phospholipids [12]. Many new calmodulin and phospholipids interacting proteins, with common potential binding motifs, such as integral membrane proteins, peripherally-associated proteins and many others, were identified. This study has demonstrated that a comprehensive set of individual proteins can be directly screened *in vitro* for a wide variety of activities, including protein-drug interactions, protein-lipid interactions and enzymatic assays, using the microarrays.

CONCLUSION

As a solution in genomic and post-genomic research fields, microarray technologies have been very dynamic and fast growing with the development of DNA microarray technology, and have also been applied to the protein chip technology. As the DNA microarrays suited the needs of a genomic approach, through high-throughput detection, protein arrays are expected to facilitate post-genomic research, such as protein identification, quantification and affinity studies, which are crucial to the development of diagnostics and proteomics. Although enormous efforts have been undertaken to expand microarray technology beyond DNA chips, protein arrays are still in their infancy. However, there are increasing needs to develop rapid, simple, cost-effective and sensitive analytic devices to facilitate protein analyses. In the future, technical developments in immobilizing proteins in their native conformation on arrays, and for more sensitive detection methods, will facilitate the rapid deployment of protein arrays as high-throughput protein assay tools in the fields of proteomics and diagnostics.

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