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Review

Biomedical applications of protein chips

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Abstract

The development of microchips involving proteins has accelerated within the past few years. Although DNA chip technologies formed the precedent, many different strategies and technologies have been used because proteins are inherently a more complex type of molecule. This review covers the various biomedical applications of protein chips in diagnostics, drug screening and testing, disease monitoring, drug discovery (proteomics), and medical research. The proteomics and drug discovery section is further subdivided to cover drug discovery tools (on-chip separations, expression profiling, and antibody arrays), molecular interactions and signaling pathways, the identification of protein function, and the identification of novel therapeutic compounds. Although largely focused on protein chips, this review includes chips involving cells and tissues as a logical extension of the type of data that can be generated from these microchips.

Keywords: protein chips - arrays - microfluidics - lab-on-a-chip - immunoassays - diagnostics - drug screening - drug discovery - proteomics

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Introduction

During the last decade, DNA chips have been powerful tools in providing a global perspective of how genes are turned on and off and as a diagnostic tool for detecting disease, leading to the transformation of biomedical research. Due to the relative simplicity of nucleic acid chemistry and genes, the manipulation of DNA chips has been relatively straightforward in spite of earlier skepticism. However, proteins are the major executors of biological function and are the targets of most drugs in the market today. From a relatively few coding genetic elements, a vast variety of proteins can be produced and have been estimated to be in the millions. This complexity of proteins in number and physiochemical properties provides the organism functional diversity in accomplishing complex biological processes for adaptation and survival. Thus adoption of a protein chip concept to understand the global expression profile and detection of proteins in different physiological states will accelerate better understanding of biology and disease mechanisms that can lead to more effective therapeutic strategies.

This review describes the different biomedical applications of a variety of protein chips and chips involving cells and tissues. Although the different protein chip formats [1-3], and fabrication technologies [4-12] are not described, it is noteworthy to mention that a protein chip system faces challenges that include the attachment of proteins in their proper conformations [7, 9, 11, 13-17], the production of stable capture agents that selectively and uniformly bind to target proteins under a defined set of conditions on the chip surface [7, 16, 18-25], the alternative use of molecular tags that resolves steric problems or inaccessibility of binding sites [26-31], expression and purification of proteins in their native conformations in high-throughput [32, 33], and the detection of protein interactions [3, 11, 12, 18-20, 34-36].

Biomedical applications

Diagnostics

Protein chips benefit clinical diagnostics and the diagnosis of complex ailments (e.g. allergies, cancer, and autoimmunity) [3]. Antibody-based immunoassays are the most common type of diagnostic assay and technologies involved with the analysis of such biomolecules develop at a rapid pace primarily because of their specificity [37]. One tool is enzyme-linked immunosorbent assay (ELISA) in a high throughput array format that can be used for detection of infectious agents or as a diagnostic for cancers [38]. Mangru and Harrison [35] performed an IgG immunoassay using chemiluminescence detection in a post-separation reactor capillary electrophoresis chip. Schneider *et al*. [20] developed an optical chip immunoassay to analyze human chronic gonadotropin (hCG) in undiluted, whole blood where better control of non-specific binding effects is expected with improved surface blocking. In human serum, the detection limit for hCG was 0.1 ng/mL [39]. A fluorescence-based immunosensor has been developed for the simultaneous analysis of multiple analytes [40]. Recognition elements on the chip capture the analytes in a given sample and detection employs a small diode laser, CCD camera, and image analysis software to correlate the positions of fluorescent signal and analyte identity. Although only spiked samples were tested, they detected physiologically relevant concentrations of staphylococcal enterotoxin B (SEB), F1 antigen from *Yersinia pestis*, and D-dimer, a marker for sepsis and thrombosis.

Competitive immunoassays can also be performed on chip. A competitive immunoassay of the drug theophylline in human serum involved an immunoreactor with subsequent capillary electrophoretic separation on the chip and fluorescence detection [41, 42]. Chromatography-type competitive assays in both single and sequential addition modes are possible. Chip layouts and the operation sequence for a 2-step competitive assay have been described [43].

The use of photo-aptamers and photo-SELEX (photochemical systematic evolution of ligands by exponential enrichment) in molecular diagnostics has powerful potential. Aptamers can be selected with high affinity (1 pM to 1 nM) and specificity for target proteins [44]. Meanwhile, the advantages of photo-aptamers are in the circumvention of the problems in array detection systems, namely, non-specific binding and low sensitivity. By a sequence of washing, photo-cross-linking, and vigorous washing (hot detergents and denaturants), non-

specific binding is reduced. Since aptamers as capture agents are oligonucleotides and differ in properties from the target molecules, proteins, the use of a universal protein stain makes the readout straightforward. A diagnostic system has been described [24].

In the discovery of prognostic and diagnostic biomarkers, sera and other body fluids, both in normal and diseased states, are profiled to identify novel biomarker candidates. Protein arrays are used to measure the abundance of specific proteins and characterize patterns of up to thousands of proteins for research or clinical applications. Haab *et al*. [45] tested the specificity and sensitivity of their assay with 115 antibody/antigen pairs in complex solutions using quantitative comparative fluorescence. Of all the arrayed samples, 50% of the antigens and 20% of the antibodies gave specific and accurate results on their binding partner at or below 0.34 μg/mL and 1.6 μg/mL, respectively. This means the sensitivities are sufficient for many clinically relevant proteins in blood samples, even more so since antibodies used in clinical diagnostics have much higher affinities than those used in research. The concentration ranges for relevant proteins were cited.

In the area of disease diagnosis, the types of leukemias are differentiated by particular subsets of the 247 cluster of differentiation (CD) antigens on the plasma membrane. Diagnosis currently involves a combination of morphology, immunophenotype, cytochemistry, and karyotype. Flow cytometry is constrained in analyzing up to 3 CDs in any one assay but using an antibody array, 50 or more CD antigens on leukocytes or leukemia cells can be analyzed [46]. Results compare well with those from flow cytometry. This allows for extensive immunophenotyping, and the intact cells that bind to the arrayed antibodies can be further characterized.

For point-of-care diagnostics, array formats have to be further reduced in size. If molecules have to travel a distance in the order of millimeters, i.e. in a microtiter well plate, incubation times will take several hours because it will take most analytes that long to diffuse throughout the entire well volume. This problem is seemingly more complicated by the fact that automation equipment (e.g. fluid handling/dispensing systems or robots) is bulky. The solution may lie in microfluidics. Since assays are done in a flowing system (non-equilibrium conditions), analysis times can be short, i.e. less than 5 min [43]. This is a major factor in considering a microfluidic platform for automating heterogeneous immunoassays. Antibodies have been immobilized directly onto fused-silica capillaries in a technique called immunoaffinity capillary electrophoresis [47, 48].

Diagnostic chips based on dielectrophoresis technology have been proposed [49, 50]. Dielectrophoresis is particle motion caused by polarization effects in non-uniform electric fields, i.e. AC rather than DC fields as in the case of electrophoresis. This technique is gentler on cells than DC techniques according to D. Jed Harrison (University of Alberta, Edmonton). The chip can, in principle, separate bacteria, fetal cells, or cancer cells from blood so infections and diseases can be diagnosed in minutes [1]. It is capable of single particle detection but at least 10 are preferred for better statistics. Organisms can also be tagged with antibodies coated on polystyrene beads. Their application is also expected to extend beyond the medical field into food and water safety testing.

Another chip, the T-sensor, can perform analyte separation and detection from complex solutions. Only minute sample amounts are required in both immunoassay and kinetic assay formats. Several clinical parameters can be tested such as blood pH and oxygen level, and detection methods range from fluorescence and light absorption to voltammetry [1].

Lastly, the DIA/PRO™ BioChip eliminates the need to centrifuge samples [51]. Blood, urine, and feces require no prior sample preparation and can be tested directly. The BioChip can perform single or multiple assays and is then inserted into an electronic reader for full quantitative analysis in minutes. The chip can run diagnostic tests for Alzheimer's disease (apo E4), many cancers, bacterial and viral infections, heart attack, and stroke [51]. They also have tests for food and water safety.

Autoimmune diagnostics profile the presence or absence as well as specificity of antibodies involved in autoimmune diseases. An example is the unambiguous diagnosis of systematic rheumatic disease [52].

Drug screening and testing

Protein chips are well suited for this application when there is only a limited supply of cells or amount of drugs available for testing [53], especially in the high-throughput screening of lead compounds. Zhu *et al.* [16] used a microarray of an entire eukaryotic proteome to screen different biochemical activities. Such microarrays can be used to screen protein-drug interactions. To identify and validate lead compounds, in vitro cellular screening is necessary before further testing in, *e.g.*, animal model [53]. Multiparametric cell monitoring with the Cell-Monitoring-System CMS® may detect side effects more easily [54,55]. Different sensors on the chip monitored different parameters. Cellular oxygen exchanges would indicate mitochondrial and photosynthetic activity; changes in cell adhesion or morphology would correspond to physiological responses; and extracellular acidification rates were monitored to determine metabolic activity. Fast pH changes (minutes) would correspond to cellular activation events such as receptor mediated signaling while slow changes (hours or longer) apply to cell proliferation or death. The signals from the different sensors were correlated to give a proper interpretation of the cellular response to a particular drug. Relative changes in cellular behavior were observed *in situ* which is an advantage over endpoint methods and which makes direct cell number determination less relevant. Furthermore, this system may be helpful in cellular pharmacokinetics, i.e. in analyzing drug action from the onset to reversibility of effects. With cell-based assays, the situation is simplified since metabolic activity does not play a role. Nevertheless, there is the restriction that only adherently growing cells can be used. After the successful testing of this sensor chip, an array format is a logical extension for high-throughput screening. The major hurdles would be in managing a more complex fluidic system and having appropriate data acquisition and processing [53].

Considering pharmacogenomics, if drug response is influenced by genetic factors, disease-specific protein chips can be used to determine whether individuals will respond favorably or adversely to a given drug. Some may not even show a response due to a defect in the drug target. This information is relevant and should be exploited for the development of new therapeutics, as well as the interpretation of clinical trial results and even treatment [56].

Disease monitoring

In monitoring diseases, identifying and detecting the presence or absence of certain proteins, i.e. protein expression profiling in normal vs. diseased tissue, is necessary. Organ and disease-specific protein arrays can be used to identify and monitor disease-related proteins using antibodies or other capture molecules [56]. As disease progresses, specific proteins can be quantified in terms of abundance, localization, and modification according to Gavin MacBeath (Center for Genomics Research, Harvard University) [7].

On a large scale, arrays can provide the means by which the patterns and variations of hundreds and thousands of proteins can be characterized for clinical or research purposes [45]. Hence, there is overlap with applications in diagnostics and drug discovery. This information can be obtained with cDNA expression libraries as a source of recombinant proteins, since these libraries represent the mRNA composition of a given sample (*e.g.* a developmental stage, cell type, or tissue) [56-59].

By studying protein regulation and expression, clinicians and researchers alike can predict predisposition to disease [56]. Once the disease is manifest, data gathered can be used for monitoring disease progression, determining response to treatment, and providing overall prognosis. It is also possible to screen for molecular markers and diagnostic and/or therapeutic targets in patient-matched tissue during disease progression [60].

Paweletz *et al.* [60] used protein arrays to support a hypothetical model of prostate cancer progression. The researchers needed to gain insight into certain molecular events in how the disease progressed in individual patients. Whole protein lysates obtained from laser capture microdissection of histopathologically relevant cells were immobilized in reverse-phase arrays. They associated cancer progression with increased Akt phosphorylation, decreased ERK (extracellular signal-regulated kinase) phosphorylation, and the suppression of apoptosis pathways. They expect that protein arrays will be used in the high-

throughput analysis of the proteomic changes in tissue cells before and after treatment of the disease.

Kononen *et al*.[61] used tissue arrays to do molecular profiling of tumor specimens obtained from 1000 tissue biopsies. This involved the simultaneous analysis of samples from many different patients at different stages of the disease. Englert *et al*.[62] also used tissue arrays in rapid molecular profiling of tumor samples.

Proteomics and drug discovery

Clearly, there is an overlap in the applications for protein arrays in the areas that benefit from the information generated. By monitoring disease-related proteins, not only can the progression of diseases be determined but also provide possible diagnostic markers and therapeutic targets [60]. Profiling and cataloging proteins in normal and diseased tissue constitute only one aspect of proteomics: expression proteomics [63, 64]. A significant portion of proteomics research lies in investigating molecular interactions and establishing the proteins that are involved in signaling pathways. All this is to gain insight into biological processes, which is an end in and of its own. However, in pharmaceutical research, to identify or validate protein function is a preliminary step in discovering novel diagnostic and therapeutic drug targets. Protein arrays help to identify disease-related proteins, or better still if possible, determine their function. Last but not least, protein arrays also aid in identifying novel, therapeutic compounds.

Drug discovery tools

On-chip separations

First and foremost, protein chips provide the tools that accelerate proteomics research. One such tool is on-chip electrophoretic separations. A commercially sold lab-on-a-chip does electrophoresis on 10 protein samples (cell lysates, column fractions, or purified proteins) in under 30 min (Fig. 1). The chip reader and software are linked so that the separations can be viewed in real time [65]. An integrated microfluidic system that incorporates protein digestion and peptide separation for protein identification has also been developed [66]. The chip contains a trypsin membrane reactor for rapid protein digestion. The resulting peptides are then concentrated and separated by capillary isotachophoresis (CITP)/capillary zone electrophoresis (CZE) prior to mass spectrometric analysis. A two-dimensional capillary electrophoresis chip has also been designed but has not been used [67]. In another application, on-chip electrophoretic separation has also been used to assess the result of a kinase A enzymatic assay where on-chip dilution of the peptide substrate was also performed to determine kinetic constants [68]. An electrophoretic separation completed in less than a millisecond has been carried out [69]. Such a separation may be useful for ultrahigh throughput drug discovery, in monitoring millisecond time-scale kinetics, or as a final clean up or separation step, e.g. in multidimensional separations. Many other electrophoretic and some chromatographic on-chip applications have been cited in the literature [12, 13, 70-73].

Fig. 1 Microfluidic channel system of the Agilent lab-on-a-chip for protein analysis. Reprinted with permission [65]. ©Agilent Technologies.

Mass spectrometry is an important detection tool for protein chips. In particular, SELDI mass spectrometry and surface plasmon resonance-biomolecular interaction analysis (SPR-BIA) provide solutions in proteomics. Although applications and formats vary somewhat, both can monitor intermolecular interactions and help to identify the interacting partners [74-78].

Expression profiling

Currently, the combination of 2D-electrophoresis and mass spectrometry can study only a limited number of proteins. Protein profiling arrays can complement 2D-electrophoresis, and in the future, possibly even replace it [19]. They can be a general resource for expression and cell map proteomics [63, 64], i.e. in high-throughput screening of gene expression and receptor-ligand interactions [3, 58, 59]. Initial screening of gene expression profiles via cDNA arrays is rapid but other methods are required to validate whether differences in hybridization signals actually correlate to physiologically relevant differences in gene expression [79]. From running the DNA arrays to profiling proteins encoded by differentially expressed cDNA clones requires a high-throughput approach to parallel protein expression analysis. This implies expressing a large number of cDNA clones simultaneously, having the appropriate vector system to do this, and arraying the resulting proteins rapidly [3, 16]. DNA sequence information and data from protein expression can be linked. Likewise, the data from DNA chips can be correlated to profiles obtained from 2D electrophoresis and mass spectrometry [56]. An alternative method is to have live cell microarrays that express the cDNA of interest and have the cells directly screened [36, 80]. As mentioned under Diagnostics, molecular profiling of tumor samples has also been done with tissue arrays [61, 62].

Antibody arrays

An offshoot of molecular profiling is antibody specificity screening against whole libraries of proteins. Since antibodies may cross-react with unrelated proteins, protein arrays would help in determining antibody specificity. However, determination of cross-reactivity using protein chips is limited by the number and type of proteins layered on the chip. Specificity screening at high throughput was done with 92 expression clones from the human fetal brain cDNA expression library, hEx1, using selected monoclonal antibodies and scFv fragments from phage display [59]. The purpose was for quality control against cross-reactivity as well as for detecting common epitopes. This reverse antibody array would be particularly important for reagents used in immunohistochemistry and physiological studies on whole cells or tissues.

Walter *et al.* [3] described the first use of a high-density protein filter. They screened the hEx1 cDNA library array with monoclonal antibodies against mouse GAPDH (glycerol aldehyde phosphate dehydrogenase), the heat shock protein 90-alpha (HSP90α), and tubulin-alpha. Holt *et al*.[81] screened protein array filters of hEx1 with antibody fragments to generate specific antibodies without the need to immunize or resort to display methods.

Although 2D gel electrophoresis is currently widely used to profile protein expression and detect protein modifications [63], antibody arrays can also be used to monitor the expression of one or more proteins in a mixture such as a cell extract [18]. Furthermore, these arrays can be used in detecting posttranslational modifications or smaller molecules, such as peptide hormones [82,83] and carbohydrates [84,85].

Detection sensitivity with antibody arrays though needs to be improved even a thousand times. scFv multimerization [86,87] may help improve detectability and biotin tyramine [88,89], *e.g.*, can be used to amplify signals.

Molecular interactions and signaling pathways

Studies on molecular interactions include peptide-protein, protein-protein, and protein-drug interactions but can also extend to epitope mapping [56]. Ge [90] did early research in this area where he used a low-density universal protein array (UPA) system to screen the interactions of p52 against 48 purified proteins spotted in nitrocellulose. His system can also be used to study protein interactions with DNA, RNA, and other ligands as well as small chemical entities. MacBeath and Schreiber [11] studied the limit of their system (FKBP12-rapamycin-binding domain) and found specific binding was detected using concentrations

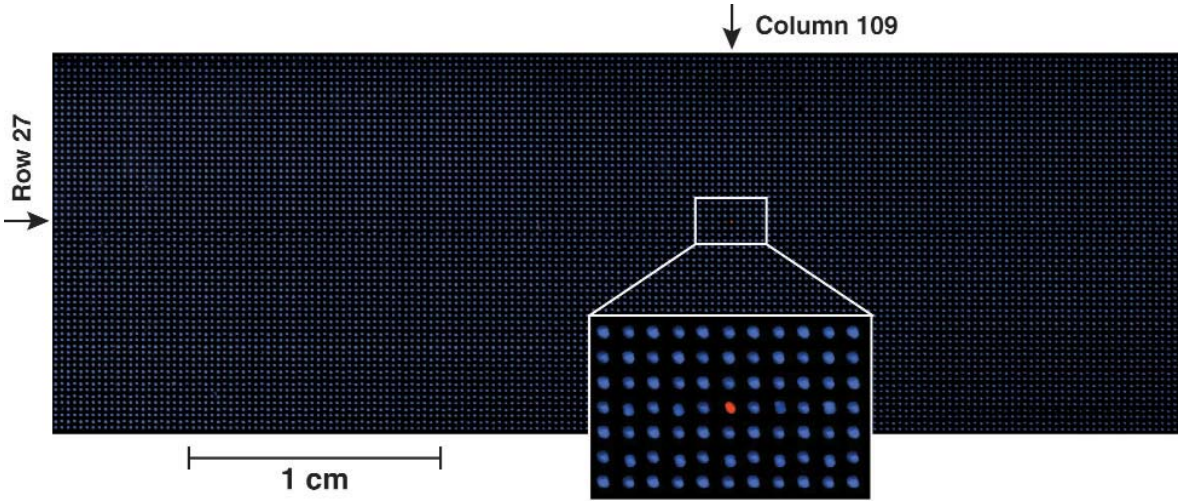

Fig. 2 A single spot of FRB(human immunophilin, FKBP12, rapamycin binding domain), in red, on a protein array with 10 800 elements. All the other 10 799 spots are protein G. Figure adapted from [11].

as low as 150 pg/mL (approx. 12.5 pM). This implies being able to find such interactions in cell lysates when using fluorescently labeled proteins. Their microarray also worked in detecting transient signals, e.g. in enzyme-substrate interactions with kinases. Protein arrays can be made with high density and specific interactions that can be detected (Fig. 2). More recently, Zhu *et al*.[16] studied interacting proteins using a yeast proteome microarray.

The study of multiprotein complexes is an extension of interaction studies. It is important for understanding cellular processes [56] and can be the basis for studying signal transduction. Signaling involves post-translational modifications, e.g. phosphorylations, which cannot be accessed by DNA methods. In particular, live cell microarrays can be used to study signal transduction because protein complexes are formed in stimulated cells so they cannot be studied in *in vitro* cell-free systems. Signaling can be induced by a number of stimuli and assays are available in different formats. Examples with JNK and p38 kinases were presented [36]. A kinase chip specifically for evaluating the function of protein kinases in cell signaling is discussed below under Identifying Protein Function.

There have been studies done on protein interactions and epitope mapping with synthetic peptides [91-93]. Geysen *et al*. [91] used a peptide array to identify an immunogenic epitope preset on the VP1 coat protein of the foot-and-mouth-disease virus.

With the use of peptide arrays, interestingly, a "spot" synthesis method, which involves the derivatization of Fmoc-β-alanine groups by hydroxyl groups on cellulose, reduces non-specific interactions [92,93]. It also increases binding affinity because of high local peptide density on the cellulose. Advantages therefore are that low-affinity antibody interactions or receptor contact sites can be determined [94].

Identification of protein function

The impetus for molecular profiling is the identification of disease-related proteins and, ultimately, the discovery of novel drug targets and prognostic and diagnostic markers. One possible strategy is to have organ and disease-specific protein arrays [56]. However, a disease-associated protein does not automatically imply an appropriate drug target or biomarker. Since molecular profiling catalogs the up- and down-regulation of proteins, the functional relevance for any one of these over- or under-expressed proteins can and should be determined or verified by another means. The probability of functional relevance increases if this

protein's participation in a signaling cascade is established. The definitive criterion for function should then be the demonstration of direct causative role of the protein in the prevailing condition or disease [64].

Various types of functional studies have been carried out on protein arrays. Live cell arrays are appropriate for large-scale characterization of protein function [36]. On the other hand, kinase chips are specifically used to determine function in the context of cell signaling, to evaluate substrate selectivity, to identify physiologically relevant enzyme-substrate pairs, and to discover novel inhibitors [95]. One kinase chip indirectly addresses function of 119 yeast protein kinases with a wide variety of substrates [16]. MacBeath and Schreiber [11] described the first attempt to develop microarrays to study protein function. Their major efforts involved immobilizing functionally active, folded proteins and detecting the interactions. They alluded to the hope of assigning function at a broader level, running miniaturized assays in parallel. Whether it is necessary to express and purify thousands of proteins or to carry out functional assays in specific cellular systems or with cell lysates, remains to be seen.

Identification of novel therapeutic compounds

The use of protein arrays to identify and validate novel therapeutics necessitates the development of assays. For example, besides identifying relevant enzyme-substrate pairs, new kinase inhibitors can be discovered using protein chips [95]. Some commercial entities intend to sell arrays containing whole protein families for use in drug lead optimization [96]. Before actual optimization studies begin, the viability of a compound as a drug lead, is determined from its binding kinetics to a specific protein drug target [51]. SPR-BIA technology can be used for this purpose. A commercial collaboration intends to carry out SPR-BIA technology in an array format for the next step, characterizing lead compounds via ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) assays [51]. All this, however, is in the near future, at the very least.

Medical research

The application of protein chips, or more broadly, biochips is accelerating medical research and providing information that was inaccessible prior to the use of this technology. The use of tissue arrays and layered expression scanning has provided researchers and clinicians with a molecular profile for each cell type [62]. On the one hand, this has immediate implications for disease monitoring and molecular profiling for drug discovery. On the other, it paves the way for a better understanding of human biology.

A true example where arrays are used in medical research is the study of electrophysiological parameters that are influenced by neurotoxicity in the brain. In the future, this research can help in understanding how electrophysiological activity is developed or organized in the brain. It can also lead to screening methods that detect toxic effects that lead to neurodenegeneration. Rat hippocampal and corticostriatal brain slice cultures are grown on perforated silicon in microelectrode arrays [97, 98]. The neurons in culture developed normally and showed the susceptibility to the neurotoxin trimethyltin (TMT) and the excitotoxin NMDA (*N*-methyl-D-aspartate) as would neurons in vivo.

Wider use of biochips in medical research can be expected as the chip technology becomes more accessible either through literature or commercial tools.

Conclusions

In spite of the infancy of protein chips and the technical challenges that remain, the applications of proteins chips in a variety of biomedical applications has been impressive. As more proteins are identified and functionally annotated, the content of the chips will increase. Technical advances in layering proteins in their native conformation on chips and development of more sensitive detection methods will facilitate the rapid deployment of protein chips in diagnostics and drug discovery. In parallel, many more biomedical applications will be conceived and realized in near future.

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