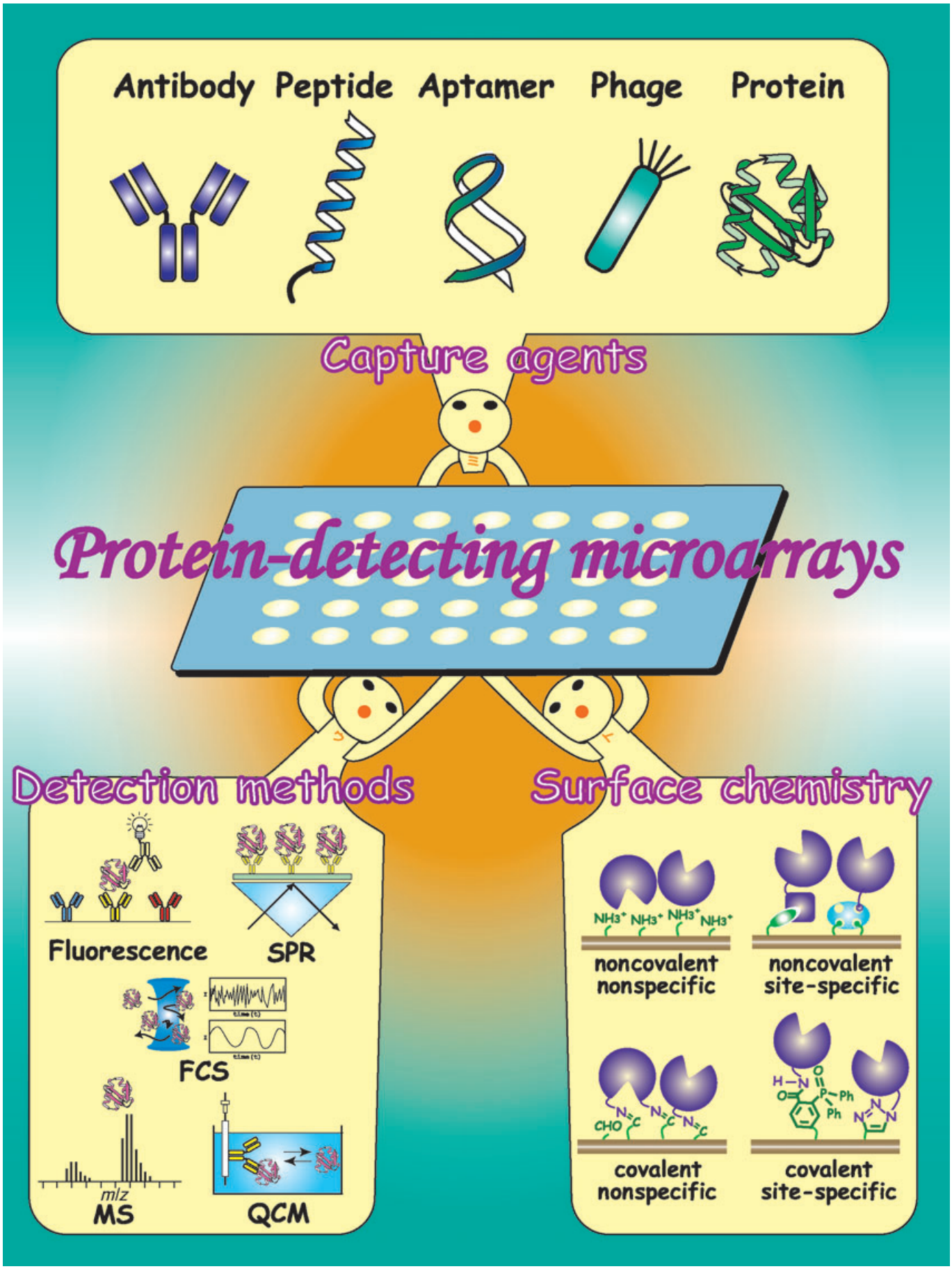

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Protein-Detecting Microarrays: Current Accomplishments and Requirements

Kin-ya Tomizaki, Kenji Usui, and Hisakazu Mihara*[a]

The sequencing of the human genome has been successfully completed and offers the chance of obtaining a large amount of valuable information for understanding complex cellular events simply and rapidly in a single experiment. Interestingly, in addressing these proteomic studies, the importance of protein-detecting microarray technology is increasing. In the coming few years, microarray technology will become a significantly promising and indispensable research/diagnostic tool from just a speculative technology. It is clear that the protein-detecting microarray is supported by three independent but strongly related technologies (surface chemistry, detection methods, and capture agents). Firstly, a variety of surface-modification methodologies are now widely available and offer site-specific immobilization of capture agents onto surfaces in such a way as to keep the native conformation and activity. Secondly, sensitive and parallel detection apparatuses are being developed to provide highly engineered microarray platforms for simultaneous data acquisition. Lastly, in the development of capture agents, antibodies are now probably the most prominent capture agents for analyzing protein abundances. Alternative scaffolds, such as phage-displayed antibody and protein fragments, which provide the advantage of increasing diversity of proteinic capture agents, however, are under development. An approach involving recombinant proteins fused with affinity tag(s) and coupled with a highly engineered surface chemistry will provide simple production protocols and specific orientations of capture agents on the microarray formats. Peptides and other small molecules can be employed in screening highly potent ligands as well as in measuring enzymatic activities. Protein-detecting microarrays supported by the three key technologies should contribute in accelerating diagnostic/biological research and drug discovery.

1. Introduction

The sequencing of the human genome has been successfully completed, thereby allowing us to obtain a large amount of valuable information for understanding complex cellular events. In the post-genome-sequence era, microarray technology is the most promising approach that enables the large-scale analyses of whole genome/protein functions (comprehensive proteome) and/or analyses focusing on the limited functions (focused proteome) simultaneously and rapidly. One of the powerful analytical tools to address such criteria is DNA microarray technology, for which thousands of oligonucleotides are synthesized on a chip by photolithographic methods[1]or polymerase chain reaction products are spotted onto a chip[2] in order to analyze mRNA transcript levels expressed under various conditions. However, it is known that the mRNA expression level and the corresponding protein abundances (or activities) do not always correlate because of changes in translation rates and protein lifetimes.[3,4] Furthermore, the analysis of mRNA transcripts does not take into account post-translational modifications, such as proteolysis, phosphorylation, glycosylation, or acetylation, although many signaling pathways mediate such structural alterations. Therefore, the motivation to overcome such difficulties has led to the development of promising technology that can allow large-scale analysis of proteins in a parallel and miniaturized fashion.

Over the past decade, the combination of two-dimensional gel electrophoresis/mass spectrometry (MS) has been the major tool in comprehensive proteomic studies; in this process, the proteome is resolved and each spot is analyzed by MS or MS/MS. The resolution of this method is good enough to separate even protein isoforms that are modified by post-translational processes (for example, phosphorylation,[5] glycosylation,[6] and deamination[7]). There are, however, several limitations, such as 1) difficulty in automation of the processes involved, 2) difficulty in the detection of less abundant proteins, 3) low reproducibility, 4) time-consuming protocols, and 5) difficulty in separation of hydrophobic membrane proteins and basic or high-molecular-mass proteins.[8–10] Another combination for proteome study is the liquid chromatography (LC)/MS method. It is possible to combine ion-exchange, reversed-phase, and affinity-based separations to improve the resolution of each protein species. Although these two technologies theoretically offer complete coverage of the proteome, they still lack the properties of parallelization and miniaturization that are required for high-throughput screening of proteins.

In order to solve the problems listed above, an alternative technology in proteomic studies, the so-called protein microarray/chip, has emerged.[11,12] The protein microarray comprises a large number of capture agents that selectively bind to the proteins of interest on solid surfaces. We, herein, would like to define this emerging technology as the "protein-detecting microarray/chip," in which candidates for capture agents are not

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only proteins, including antibodies and native and engineered proteins, but also DNA/RNA aptamers, peptides, and other organic molecules. Therefore, when antibodies are immobilized onto the solid surface, the method should be called an "antibody microarray", and likewise when peptides are the capture agents, the technology should be called a "peptide microarray".

Protein-detecting microarrays mainly allow us to perform two different types of analyses, depending on their purposes for protein detection (Figure 1). One is to determine the abundances of proteins of interest in complex protein mixtures with highly specific capture agents for each target protein, for example, by antigen–antibody interactions. The other is to find out the functions of proteins of interest, including protein–protein interactions, receptor–ligand interactions, enzymatic activities, and so on. In general, the protein-detecting microarray is supported by three distinct technologies: surface chemistry providing highly engineered surfaces, development of high-throughput detection methods, and production of functional capture agents. These key technologies must be tied in with each other to create valuable protein-detecting systems.

As a reflection of the impact of this emerging technology in proteomic studies, dozens of exciting reviews on protein-detecting microarrays have been reported in past few years; these reviews describe the concept of such microarray technologies and focus on the production of microarrays and the development of capture agents.[13–26] Although protein-detecting microarray technology will be indispensable in proteomic studies, it seems to be still at the middle point on the way to the final destination where a tiny, widely available chip will routinely allow us to obtain thousands to tens of thousands of important parameters in a single experiment. Furthermore, review articles covering a broader range, including all surface chemistry, detection method, and capture agent issues within their content, have rarely been published, although the three technologies that seem to be independent are strongly related to each other. In this review, we focus on the features of surface chemistry for immobilization of capture agents onto the solid support, the development of detection methods, the characteristics of each capture agent, and the current accomplishments and requirements of protein-detecting microarray approaches in proteomic research from the viewpoint of chemical biology in order to understand the present situation and future of this microarray technology.

2. Surface Chemistry for Immobilization of Capture Agents onto Solid Surfaces

In order to establish reproducible and reliable protein-detecting microarrays, it is necessary to place capture agents in such a way as to maintain their active forms on a solid surface. Summarized in Table 1 are the representative methodologies that are classified into the four different categories of nonspecific/noncovalent, nonspecific/covalent, site-specific/noncovalent, and site-specific/covalent immobilization methods for flat surfaces and a wet-system for three-dimensional surfaces. The four different immobilization technologies for the flat surfaces

are also illustrated in Figure 2. We firstly describe a series of simple nonspecific immobilization techniques, in which the capture agents are attached onto 1) soft membranes such as PVDF[13] and nitrocellulose membrane,[27] and 2) glass slides modified with nitrocellulose (FAST slides)[28] or poly(L-lysine).[29] Lee et al. have also developed a highly sensitive protein-detecting microarray coated with the calixcrown-5 derivatives having two functionalities, one of which is a crown moiety that recognizes proteins through host–guest interactions of an amino group at the protein surface and the other of which is a linker moiety (formyl or thiol group) that binds to the solid surfaces through Schiff's base or mercaptide bonds onto an amino-coated or gold surface, respectively.[30]

Secondly, the nonspecific/covalent immobilization protocol employs solid surfaces modified with aldehyde,[31,32] epoxide,[33] or succinimidyl ester/isothiocyanate functionalities.[34] As another class of the nonspecific/covalent method, photoaffinity linkages have been utilized, in which photoreactive aryl diazirines have been attached onto glass slides for the photoimmobilization and proteins were printed onto the diazirine-coated surfaces with UV irradiation.[35] Such nonspecific immobilization approaches do not require any modifications of capture agents, thereby facilitating capture-agent production, but do lack defined orientations of the capture agents on the solid support.

Thirdly, site-specific/noncovalent immobilization techniques have been developed that use an affinity tag, such as the biotin moiety, His tag, or GST, at the N or C terminus of the capture agent, together with the corresponding modified surfaces of avidin-coated,[36–39] Ni-NTA-coated,[32] and glutathione-coated glass slides,[40] respectively. The use of Protein-A- or Protein-G-coated surfaces to immobilize immunoglobulin G (IgG), which is known to bind strongly to the Fc region in antibodies, would be an alternative approach.[41,42] A new class of the site-specific/noncovalent immobilization technique has emerged. Winssinger and co-workers[43–46] and Lovrinovic et al.[47] displayed a variety of molecules, including small molecules, peptides, and recombinant proteins, tethering oligopeptide nucleic acid (oligo-PNA) tags onto DNA microarrays through PNA–DNA hybridization. This can provide a chemically mild procedure for the site-specific attachment of the capture agents to the solid surface with a predetermined orientation on the microarray that suppresses the loss of their native conformations and activities.

Finally, we describe site-specific/covalent immobilization techniques. Self-assembled monolayers (SAMs) on gold surface have been widely employed as a surface-modification tool;[48] one of these monolayers comprises alkanethiols joined with oligo(ethylene

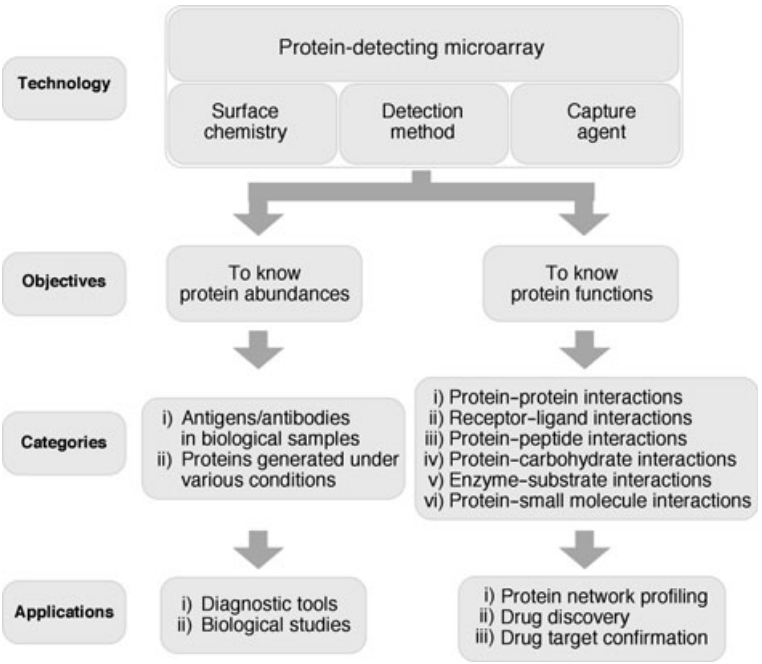

Figure 1. *Protein-detecting microarrays are supported by three technologies (surface chemistry, detection method, and capture agent) that are independent but related to each other. These microarrays mainly offer two different types of analyses, one of which is to know protein abundances in complex protein mixtures from various biological samples and the other of which is to know protein functions involving molecular recognition and enzymatic activities. These microarrays are applicable for use in diagnostic tools and biological studies, as well as for protein network profiling, drug discovery, and drug-target confirmation.*

Table 1. *Surface modifications that would be applicable in the development of protein-detecting microarray technology.*[a]

Categories	Surfaces (modified with)	Capture agents (with)
Flat surfaces:		
Nonspecific/ noncovalent ways:	PVDF	functional group-independent
	nitrocellulose	functional group-independent
	poly(L-lysine)	functional group-independent
	calixcrown-5 derivatives	amines
Nonspecific/ covalent ways:	aldehyde	amines
	epoxide	amines, thiols
	succinimidyl ester/ isothiocyanate	amines
	photoaffinity reaction	functional group-independent
Specific/ noncovalent ways:	avidin	biotin tag
	Ni-NTA	His tag
	glutathione	GST tag
	protein A/G	IgG Fc region
	oligoDNA	oligoPNA
Specific/ covalent ways:	maleimide	thiol group
	bromoacetyl	thiol group
	thioester	cysteine at N terminus
	glyoxylyl group	aminooxy acetyl group
	semicarbazide	glyoxylyl group
	Diels–Alder reaction	
	1,3-dipolar cycloaddition	
	Staudinger reaction	
Three-dimensional surfaces:	agarose/polyacrylamide gel pad	
	PDMS film	
	supramolecular hydrogel	

[a] Abbreviations: PVDF = poly(vinylidene difluoride), Ni-NTA = nickel(II) nitrilotriacetic acid, GST = glutathione-*S*-transferase, PNA = peptide nucleic acid, PDMS = polydimethylsiloxane.

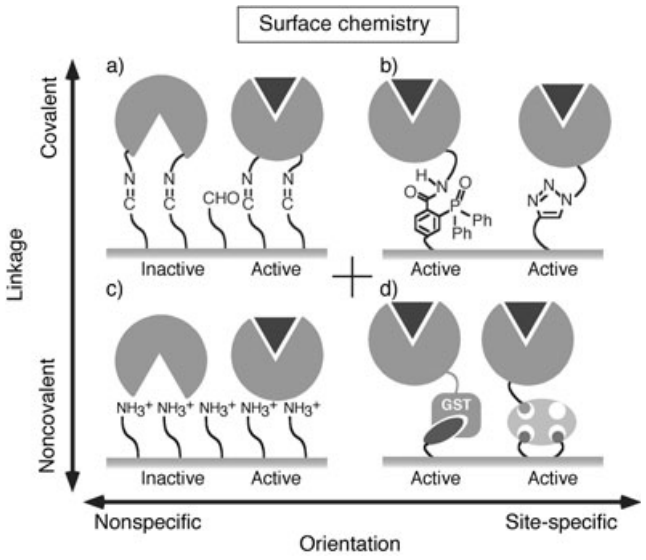

Figure 2. *Classification according to surface attachment of the capture agents. a) Nonspecific/covalent attachments through a Schiff's base, which is formed with aldehyde/amino groups, allow an inactive conformation of a fraction of the capture agents attached on the surface. An alternative random/covalent approach with epoxide/thiol (amino) groups or maleimide/thiol groups is also available. b) Site-specific/covalent attachments produced through a Staudinger reaction (left) and 1,3-dipolar cycloaddition (right) are formed with triphenylphosphine/azide groups and alkyne/azide groups, respectively. Such prominent approaches, however, are rare due to difficulties in site-specific incorporation of azide-derivatized amino acid residues into the capture agents. c) Simpler nonspecific/noncovalent attachments through physical/chemical adsorption, including electrostatic, hydrophobic, and van der Waals interactions, are widely employed but generally lack site-specific immobilization of the capture agents. Nitrocellulose-coated or poly(L-lysine)-coated slides are also available. d) Site-specific/noncovalent attachments through glutathione/glutathione-S-transferase (left) or avidin/biotin (right) affinity hybridizations offer efficient fabrication of the protein-detecting microarrays together with the improvement of recombinant-protein production protocols. His-tagged molecules can also be attached onto the Ni-NTA-modified surfaces site-specifically.*

glycol) groups known to suppress nonspecific adsorption of proteins to the surface.[49] A fraction of one end in the oligo(ethylene glycol) groups is modified with a benzoquinone functionality that makes a covalent bond with cyclopentadiene-containing peptides through the Diels–Alder reaction. This surface chemistry has been employed in assessing enzymatic phosphorylation by a protein kinase,[49] a process that clearly demonstrated that chemical ligation at the solid surface can preferentially control the configuration and density of the capture agents. Monolayers of derivatized poly(L-lysine)-grafted poly(ethylene glycol) (PEG) should also be effective as biomolecular interfaces that suppress nonspecific bindings and offer defined orientations.[50] Thiol-derivatized sugars have similarly been immobilized onto SAMs on gold through thiol–maleimide conjugation.[51] Synthetic peptides containing a free cysteine amino acid residue were also immobilized covalently onto plastic surfaces modified with bromoacetyl groups through the layers of poly(L-lysine) and dextran coatings to detect protein–peptide interactions in an array format.[52,53] Chemical ligation of N-terminal cysteine-containing biomolecules to slides containing thioester moieties has also been successfully performed.[54] Although these are quite useful methods to attach synthetic peptides and sugars to the solid surfaces, there are still bottlenecks in immobilizing proteins, due to the characteristic localization of free cysteine residues in each protein.

As an alternative approach, glyoxylyl-peptides have been immobilized onto semicarbazide-functionalized glass slides through α-oxo semicarbazone linkages to exhibit highly sensitive and specific properties for the detection of antibodies in human sera.[55,56] There are some other exciting chemical ligation systems that would be applicable for creating highly engineered solid surfaces, namely, [1,3]-dipolar cycloaddition[57,58] and Staudinger ligation.[59] The exclusive feature of these chemical ligation techniques is the lack of cross-reactivities of functional groups in the reactants with any amino acid side chain at the surfaces of naturally occurring proteins; this feature is of benefit in site-specific immobilization of capture agents onto the substrates. The former ligation system, involving the covalent cycloaddition of azides and alkynes catalyzed by copper(I) to form (1,2,3)-triazoles, has been successfully demonstrated in the modification of the surface of Cowpa mosaic virus (CPMW) with a fluorescent dye molecule.[58] The latter system involved the incorporation of azide-functionalized amino acids (for example, azidohomoalanine as a methionine analogue) into murine dihydrofolate reductase (mDHFR) by using methionyl-tRNA synthetase (MetRS), followed by Staudinger reaction of azidohomoalanine in mDHFR with the triphenylphosphine-conjugated FLAG peptide within a complex cell-lysate mixture.[59] Although both [1,3]-dipolar cycloaddition and Staudinger reactions do not directly overcome the difficulties of immobilizing the proteinic capture agents site-specifically with defined orientations at the present stage, such chemically developable approaches might provide highly engineered surfaces that would possibly be helpful in expanding the diversity of protein-detecting microarray formats, together with the establishment of efficient methods for the functionalization of capture agents.

Three-dimensional platforms have been also developed; they are the gel pad,[60] agarose and polyacrylamide gel pad,[61,62] sol-gel-encapsulated biomolecules patterned within multiwell PDMS films,[63] and supramolecular hydrogel methods.[64] Such a homogeneous aqueous environment prevents protein denaturation more effectively than is possible in proteins attached on the flat surfaces, but it is difficult to change solvents inside the gel formation.

As further applications of surface chemistry, some exciting methods have been reported for the immobilization of membrane proteins onto the chip substrates in such a way as to keep their activities and native conformations intact. Bieri et al. reported an approach to immobilize G-protein-coupled receptors (GPCRs) stably and with defined orientation onto sensor chips that were covered with mixed SAMs consisting of biotinylated thiols and an excess of ω-hydroxy-undecanethiol, to which streptavidin was bound.[65] The biotinylated receptor was then immobilized onto the chip through the biotin–streptavidin binding. Fang et al. also developed methodologies in the fabrication of membrane-protein microarrays consisting of GPCRs immobilized onto a γ-aminopropylsilane (GAPS) coated

surface in which membrane microspots on GAPS were obtained by printing vesicular solutions of dipalmitoylphosphatidylcholine (DPPC)/dimyristoylphosphatidylcholine (DMPC; 4:1) or egg-yolk phosphatidylcholine (PC) doped on glass slides.[66,67]

Very recently, Urbanowska et al. developed antibody microarray technology to monitor biomarkers of rheumatoid arthritis disease.[68] In order to develop a high-performance antibody microarray system, surface-modification methods were first investigated. Novartis proprietary glass chips were coated with an octadecyl phosphoric acid (ODP) SAM formation or with poly(L-lysine) and antibodies were deposited onto the chips by a contact printing or a noncontact piezoelectric dispensing technology. As a result of this study, a combination of the ODP SAM surface and the noncontact piezoelectric dispensing method was found to be preferable to other conditions due to the lower background noise with respect to the surface chemistry, reduced cross-contamination, and more accurate and precise spotting.

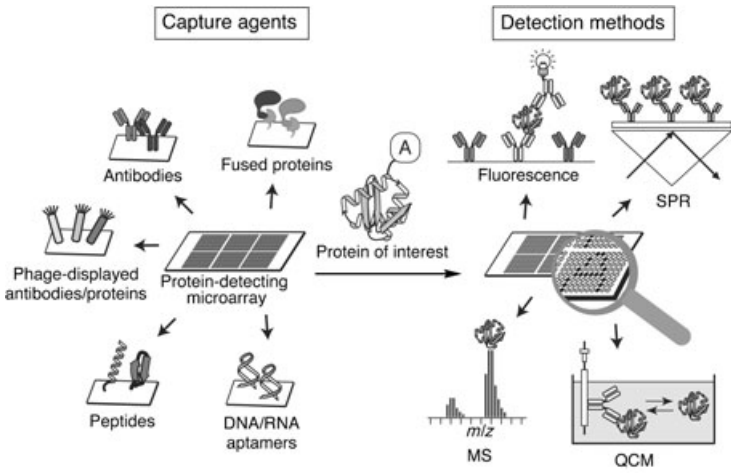

Figure 3. *Classification of representative protein capture agents and detection methods applicable for the development of protein-detecting microarray technology. At the present stage, it is difficult to discuss which capture agent or detection method is clearly the most suitable for the microarray format. Each candidate has a unique pattern of potential applications.*

As described above, although there are a number of choices to fabricate microarray platforms that have been successfully employed in proteomic studies, we are still not able to discuss which surface chemistry is the most suitable for the criteria of time consumption, cost performance, and reproducibility. We need more time and experiments to create highly engineered surfaces addressing the center of immobilization technology.

3. Detection Methods

Detection methods in analyzing any array are required to offer high throughput, high signal-to-noise ratio, relatively low instrumentation costs, good resolution, and reproducible results. Although the most suitable detection method that meets such criteria is still under consideration, there are several candidates, such as 1) fluorescent labeling,[31,32] 2) isotopic labeling,[33,49] 3) chemiluminesent labeling,[69] 4) mass spectrometry,[70] 5) surface plasmon resonance (SPR) spectroscopy,[71–75] 6) anomalous reflections (AR) of the gold surface,[76] 7) quartz-crystal microbalance (QCM) analysis,[77–79] 8) fluorescence correlation spectroscopy (FCS),[80–82] and 9) electrochemical detection (Figure 3). It is necessary to label with fluorescent probes for the methods 1 and 8, with radioisotopes for method 2, with an adequate functional group/molecule for method 3, and with electrically active probes for method 9, but no labeling at all is necessary for the other methods (4–7). The features of the different methods with respect to their availability for protein-detecting microarrays are summarized in Table 2 and discussed below.

Many applications have used fluorescently labeled detection methods, because they are simple and stable to manipulate, they provide highly sensitive and resolved results, and they are compatible with the standard array scanners developed for DNA microchips. In the sandwich assay system, capture agents such as antibodies can be attached in a defined pattern on solid surfaces. In general, the assay involves capture of the pro-

Table 2. *Detection methods that would be applicable in the development of protein-detecting microarray technology.*[a]

	Probe labeling	Quantitative analysis	High throughput	Instrumentation costs	Comments
fluorescence	yes	yes/no	yes	inexpensive	sensitive, large dynamic range
chemiluminescence	yes	yes/no	yes	inexpensive	sensitive, large dynamic range
radioisotopes	yes	yes	yes/no	medium	safety concerns
MS	no	no	no	expensive	accessing molecular mass directly, addressing post-translational modifications
SPR spectroscopy	no	yes	no	expensive	mass measurement, kinetic parameters
AR	no	yes	yes	inexpensive	mass measurement, simple optical geometry, easy to miniaturize, kinetic parameters
QCM analysis	no	yes	no	inexpensive	mass measurement, kinetic parameters
FCS	yes	yes/no	yes	expensive	single-molecule sensitivity, liquid phase (no immobilization needed)
electrochemical detection	yes/no	yes	yes/no	inexpensive	sensitive, large dynamic range, easy to miniaturize, kinetic parameters

[a] Each detection method is qualitatively represented in the following five criteria: 1) requirements of probe labeling(s), 2) quantitative identification of protein abundances and activities, 3) possibility of high-throughput analysis, 4) instrumentation costs (availability), and 5) comments on potential/current limitations.

teins of interest in a complex mixture followed by washing of the surfaces to remove nonspecifically bound species. Subsequently, a secondary antibody labeled with a fluorescent dye molecule that can recognize exposed epitopes on the bound proteins is added to quantify the results. This sandwich assay allows more increased specificity than the immunoassay based on a single antibody method, because the duplicated recognition steps with two antibodies that bind two distinct epitopes successfully reduce cross-reactivity. Furthermore, the rolling circle amplification (RCA) method has been developed to improve sensitivity in the fluorescent detection system and has been applied to detect different cytokines with detection levels of around femtomolar concentrations.[83,84] Even such a recently emerging, sophisticated binding assay system, however, still has a bottleneck, in that two distinct capture agents are required for analyzing one protein of interest; this means that if there are a thousand proteins to be analyzed, more than two thousand antibodies essentially have to be prepared in this assay method.

Although radioactivity is also suitable for analyzing arrays, especially for enzymatic phosphorylation, due to sensitivity and specificity as well as the possibility of fluorescence detection, the use of isotope-labeled molecules raises safety concerns. Therefore, this method is not likely to be adaptable for high-throughput screening. Chemiluminescence is also highly sensitive but at present gives relatively lower resolution and relatively limited dynamic range.[85] The use of both the radioactivity and chemiluminescence approaches is discussed later.

Mass spectrometry allows us to obtain direct information (molecular masses) about proteins of interest and does not require any labeling molecules, which sometimes alter the conformations and activities of the target molecules. This is preferable to the other detecting methods described above. Very recently, mass spectrometry was successfully employed to explore the phosphoproteome of a seedless plant.[70] The complex phosphopeptide mixture after tryptic digestion was separated by reversed-phase HPLC and each compound was analyzed by capillary zone electrophoresis. The 253 distinct phosphopeptides obtained were identified by nanoscale LC/MS/MS and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses, in conjunction with alkaline phosphatase treatment to remove covalently bound phosphate, in order to specifically identify the phosphopeptides. This example strongly suggests that mass spectrometry is a powerful tool for the analysis of post-translational modifications of proteins. Mass spectrometry, however, has the disadvantages of relatively difficult quantification and low throughput. Mass spectrometric techniques combined with other detection methods in parallel and/or in series might offer more powerful analysis for comprehensive proteomic studies.

Surface-enhanced laser desorption/ionization (SELDI) TOF MS is an innovative approach that offers an on-chip purification of the proteins of interest and subsequent ionization of the retained molecules to be detected.[86] Recently, the SELDI-TOF MS system has been positively employed in the detection of several disease-specific marker proteins in real biological samples, that is, to perform profiling of amyloid β peptide variants secreted from culture cells,[87] profiling of rat plasma for biomarker discovery,[88] identification of novel and downregulated biomarkers for alcoholism,[89] and molecular classification of liver cirrhosis in a rat model.[90]

SPR spectroscopy[71–75] is well-known to allow us to study kinetics of antigen–antibody, protein–protein, and receptor–ligand interactions in real time without any labeling molecule. The capture molecules are immobilized on a gold surface and unlabeled analyte is added. The change in the reflection angle of light indicates the amount of target molecules captured on the surfaces. SPR spectroscopy is a really versatile tool but enables analysis of only a few channels in a single experiment. This method requires a large number of samples to be placed on the gold surfaces for microarray formats. Very recently, the SPR imaging technique has been rather popular for the detection of molecular interactions in a parallelized fashion.[91,92] The assay format can be adapted to study hundreds of molecular interactions within a single experiment.

Recently, Watanabe and Kajikawa demonstrated a simple and sensitive optical-fiber sensing system by using AR of gold surfaces for the streptavidin–biotin interaction.[76] AR are a characteristic phenomenon of gold that behaves as a dielectric with a large extinction index under blue or violet light, because a large decrease in the reflectivity of the gold surface results when it is modified with a transparent surface layer, due to multiple reflections of the surface. The AR method has a number of advantages: 1) the optical geometry is simple, thereby facilitating miniaturization; 2) results obtained can be interpreted quantitatively by solving Maxwell equations; 3) the gold surface can be prepared by a simple vacuum-evaporation method; and 4) AR are not a resonance effect and occur over a large range of wavelengths of light (350–500 nm), thereby allowing use of incoherent light sources with a broad emission band, such as a light-emitting diode (LED). This method can offer quantitative analysis of biological events in the microarray formats and the use of inexpensive and disposable micrometer-sized probes. Optimization of the system is now in progress.

The QCM is a sensitive mass-measuring device both in air and in aqueous solutions, and the resonance frequency has been proved to decrease linearly with increasing mass on the QCM electrode at a nanogram level. The QCM method has been employed to observe biological events in real time. The QCM frequency responded to three steps in polymerase reactions, namely binding of DNA polymerase to the primer on the QCM (mass increase), elongation of complementary nucleotides along the template (mass increase), and release of the enzyme from the completely polymerized DNA (mass decrease), as a function of elapsed time.[77] In vitro selection of N-peptide-binding RNA to study a sequence-specific interaction between the peptide and loop RNA[78] and direct monitoring of enzymatic glucan hydrolysis[79] were also successfully demonstrated with the QCM method. Parallelized platforms for QCM measurement are expected to be developed.

FCS has recently experienced growing popularity in biochemical and biophysical applications due to a significantly im-

proved signal-to-noise ratio and single-molecule sensitivity not on the surface but in a quite small volume of solution ($\approx$1 fL), which can represent biological events more precisely and reproducibly than microarray formats established on solid supports.[80–82] The method is based on the recording of spatiotemporal correlations among fluctuating light signals coupled with the trapping of single molecules in an electric field. The diffusion times obtained from the fluctuating signals provide valuable information on the molecular size of the complex formed. Although the FCS assay system is expected to allow the analysis of molecular interactions on a proteome-wide scale, there are some essential requirements, in that fluorophore-labeled capture agents are necessary for monitoring and the changes in diffusion time obtained are strongly dependent upon the differences in the molecular size before and after complexation.

The electrochemistry-based detection technique has been primarily developed for analyzing DNA-hybridization events and has shown the potential to enable low-cost, real-time, and highly sensitive measurements.[93] Recently, an amperometric immunosensor based on a horseradish peroxidase (HRP)/hydrogen peroxide coupled catalytic system has been reported, in which antibodies were immobilized onto the electrode and complexation was subsequently performed with antigens modified with HRP.[94] Another study prepared an amperometric immunosensor based on the glucose oxidase (GOD)/HRP coupled catalytic system, in which antibodies were immobilized onto the HRP-coated electrodes and antigens linked with GOD were subsequently added to generate hydrogen peroxide.[95] An electrochemical protein chip with an array of 36 electrodes has been microfabricated, in which antibodies were immobilized in a plasma-polymerized film and exhibited good discrimination between α-fetoprotein and β_2-macroglobulin, by using a sandwich immunoassay technique with a GOD-modified secondary antibody.[96] In the use of electrochemical detection for biological systems, two important obstacles have been pointed out; these are 1) the use of electrochemically active agents facilitating electron transfer and 2) the development of surface-modification methodologies providing well-defined and reproducible surfaces for electrochemistry. Very recently, frequency-dependent eletrochemical impedance spectroscopy has been used to characterize the change in electrical response resulting from specific binding of a protein to its substrate in a completely label-free manner.[97] Further efforts might open the way to a versatile detection method.

We have discussed above the detection techniques to be employed in the microarray technology. The fluorescence sensing system coupled with antibodies seems to be the simplest and most versatile because it can provide highly specific and sensitive analyses. The bottlenecks, however, are the production of antibodies that can recognize only their specific antigens and the quantitative labeling of antibodies/antigens. Radioactivity is often used to screen specific substrates of protein kinases in the presence of isotope-labeled adenosine triphosphate (ATP) due to the incompatibility of phosphorylated sequence-specific antibodies in some cases. In the near future, when the production of micropatterned and reproducible surfaces are highly developed, the SPR imaging method will become one of the most prominent techniques for analyzing molecular interactions without any labeling. In order to improve the total reliability and efficiency in profiling molecular interactions on the proteome scale, high-throughput analytical protocols as a first screening can be followed by accurate analyses such as MS and SPR spectroscopy.

4. Capture Agents and Their Current Accomplishments

The selection and production of the capture agents are the most critical points in developing protein-detecting microarray technology. Antibodies and their fragments (scFv), antigens, fused proteins, DNA/RNA aptamers, peptides, sugars, molecularly imprinted polymers (MIPs), and other classes of small-molecule capture agents have been vigorously studied and immobilized onto solid surfaces. The divergent properties in the capture agents are important points for the development of each individual microarray (Figure 3). Their features for the protein-detecting microarray are summarized in Table 3. Here, we focus on the characteristics and current accomplishments and requirements of each capture agent in order to understand their potential and prospects, although some have already been mentioned in the previous sections.

4.1. Antibody/antigen microarrays

Antibodies are one of the most prominent capture agents with high affinity and specificity to target molecules. In one of the

Table 3. *Capture agents that would be applicable in the development of protein-detecting microarray technology.*

Capture agents	Production/purification	Target molecules	Objectives (to analyze)
antibodies	hybridoma cells	antigens	protein abundances
antigens	recombinant, extraction	antibodies	protein abundances
fused-proteins	recombinant, affinity purification	proteins	protein abundances/functions
phage-displayed proteins/ antibody fragments	complementary DNA library, in vitro selection	proteins	protein abundances/functions
DNA/RNA aptamers	SELEX[a]	small molecules–proteins	protein abundances
peptides	chemical synthesis	antibodies, enzymes	protein functions
carbohydrates	chemical synthesis, extraction	sugar-binding proteins	protein functions
small molecules	chemical synthesis	receptors, enzymes	protein functions
MIPs	chemical synthesis	small molecule-s–proteins	protein abundances

[a] SELEX = systematic evolution of ligands by exponential enrichment process.

most extensive studies in the early stages of protein-detecting microarray technology, Schweitzer et al. created an antibody microarray comprising of 75 antibodies against cytokines on glass slides and analyzed cytokine secretions from human dendritic cells induced with lipopolysaccharide or tumor necrosis factor-α.[83] Haab et al. immobilized 115 antigen/antibody pairs onto poly(L-lysine)-coated glass slides to create both antibody microarrays for detecting antigens and antigen microarrays for detecting antibodies, with a ratiometric, two-color labeling approach.[29] 50% of the arrayed antigens and 20% of arrayed antibodies provided specific and accurate measurements of their cognate ligands at concentrations of 0.34 and 1.6 $\mu g\,mL^{-1}$, or less, respectively. Some of the antigen/antibody pairs allowed detection of the cognate ligands at concentrations below 1 $ng\,mL^{-1}$, which is sensitive enough for diagnostics. Sreekumar et al. demonstrated the analysis of LoVo colon carcinoma cells in response to treatment with ionizing radiation by using a two-color labeling technique.[98] More than 100 antibodies against proteins involved in stress response, cell cycle progression, and apoptosis were immobilized onto poly(L-lysine)-coated or superaldehyde-modified glass slides. In this study, several potential regulatory proteins for radiation-induced apoptosis signaling in LoVo cells and a remarkable radiation-induced down-regulation of carcinoembryonic antigen (CEA), a cancer biomarker, were revealed.

One of the greatest goals of the protein-detecting microarray is analyzing changes in the abundance/existence of proteins in a very large dynamic range (factors of 10^6–10^{10}) in biological samples (biopsies, tissue-cell aspirates, or body-fluid samples) under the various conditions applicable for a diagnostic tool.[99–103] In such diagnostic applications, the use of the antigen–antibody immune response is particularly effective, due to its high specificity and affinity. Robinson et al. constructed miniaturized autoantigen arrays and demonstrated sensitive and specific detection of characteristic autoantibodies, as well as identification of the isotype of the antigen-specific antibodies. Detection of antibodies revealed the corresponding post-translational modifications in serum from patients with eight distinct autoimmune diseases.[99] Miller et al. developed a practical strategy for serum-protein profiling with antibody microarrays and applied the method to the identification of potential biomarkers in prostate cancer serum, in which antibodies were immobilized onto the polyacrylamide-based hydrogels on glass or poly(L-lysine)-coated glass with a photoreactive cross-linking layer.[100] In this study, five proteins (von Willebrand factor, immunoglobulin M, α1-antichymotrypsin, villin, and immunoglobulin G) that had significantly different levels between the prostate cancer samples and the controls were identified. The antibody microarray approaches described above enable the use of "protein-detecting chips" in diagnostic applications and biomarker discovery.

Bouwman et al. examined the use of microarrays comprising tumor-derived proteins to profile the antibody repertoire in the sera of prostate cancer patients and controls.[101] The protein mixture from the prostate cancer cell-line LNCaP was divided into 1760 fractions by two-dimensional liquid chromatography and then deposited onto the nitrocellulose-coated microscope slides. The obtained microarrays were incubated individually with cancer serum samples and controls. Significantly higher levels of immunoglobulin reactivity from the prostate cancer samples were observed, a result reflecting a strong immune response to the tumor-derived biomarker(s) in the prostate cancer patients. These results suggest that microarrays of fractionated tumor antigens could be a powerful tool for tumor-antigen discovery and cancer diagnostics. An antibody microarray approach is the most powerful tool to analyze the abundance of proteins produced in varying dynamic concentration ranges under different conditions.

Recently, Michaud et al. examined the cross-reactivities of 11 polyclonal and monoclonal antibodies against $\approx$5000 different yeast proteins deposited onto glass-slide "whole proteome microarrays", and they found that the antibodies not only recognized cognate proteins but also cross-reacted with other yeast proteins in varying degrees.[104] The proteome array approach has the potential to improve antibody microarray design and selection for application in diagnostics, because these interactions could not be predicted a priori. Indeed, although numerous antibodies have been immobilized onto the solid surfaces in the previous studies and have identified many known interactions successfully in large-scale (sometimes comprehensive) analyses, antibodies have sometimes exhibited varying performances involving no reactivity or lowered specificity/affinity.[29,104] These results suggest that the antigens/antibodies used should be validated by the standard procedures to obtain reliable capturing antibodies even on well-studied solid surfaces.

The enzyme-linked immunosorbent assay (ELISA) is typically applied in the format of microtiter plates. In order to reduce sample consumption and improve throughput, microarray-based ELISA techniques have been developed.[105–107] Angenendt et al. described an immunoassay performed on a standard microscope slide without the requirment for wells or tubes to separate the samples; this new multiple-spotting technique comprises immobilization of a capture agent onto a surface and subsequent spotting of a target protein on the same spot.[107] A careful alignment of the microchips to the grid of the spotting robot could avoid imperfect overlap of sample transfer under the same conditions.

The method of site-specific immobilization of antibodies should be developed, as well as the method for generating them. Recently, Kozlov et al. demonstrated conjugation of aldehyde-derivatized oligonucleotides and hydrazine-derivatized antibodies to form covalent hydrazone bonds which are stable over long periods of time under physiological conditions and suitable for very sensitive assays to determine protein concentrations.[108] Although the use of antigen–antibody immune responses is particularly effective due to their high specificity and affinity, the critical requirements of antibody microarrays are the establishment of selection/production/purification methods for generating antibodies with high affinity and a reduced cross-reactivity, incorporation of the probe(s) into antibody surfaces, and immobilization methods that maintain the native conformations and activities.

4.2. Fused-protein microarrays

In order to detect protein functions in a microarray format, proteins were also deposited in microfabricated polyacrylamide hydrogel pads[62] or directly immobilized onto a variety of chemically modified glass slides.[31] Although the former format can keep native proteins active on the microarray, it is difficult to fabricate and change buffers inside the gel formation, as described above, while the latter format essentially allows nonspecific attachments of proteins on the surface, probably causing them to lack native conformations and activities. The introduction of affinity tags to either the N or C terminus of recombinant proteins is a prominent technology to facilitate purification of proteins from a complex mixture, to enable site-specific immobilization of proteins onto solid surfaces with their activities intact, and sometimes to improve the solubility of the constructs. Several affinity fusions have been developed into recombinant proteins so far, for example, six histidine residues (His tag),[109] strep-tagII,[110] calmodulin-binding peptide,[111] chitin-binding protein,[112] GST,[113] maltose-binding protein,[114] and thioredoxin.[115]

Accompanying with development of these production/purification techniques for proteins, protein microarray technology, in which the affinity-tagged fusion proteins are immobilized onto the solid surface, has emerged. Zhu et al. expressed 119 yeast kinases fused with a GST tag and immobilized them covalently into microwells made by a disposable silicone elastomer, PDMS, by using a cross-linker of 3-glycidoxypropyltrimethoxysilane (GPTS).[33] By using 17 different substrates and the microarray, many novel activities involving a large number of protein kinases that were capable of phosphorylation of tyrosines were identified. Kawahashi et al. also prepared two kinds of fused proteins, GST-fused proteins and probing proteins modified with a fluorescently labeled puromycin by a cell-free protein-synthesis system, both of which were labeled with an N-terminal T7 tag for quantification and a C-terminal His-tag sequence for affinity purification.[40] They then arrayed the fused proteins onto glass slides coated with poly(L-lysine)-grafted PEG copolymers tethering glutathiones and analyzed the protein–protein interactions by adding probing proteins into the microarray format.

Weng et al. generated addressable protein microarrays with mRNA–protein fusions consisting of polypeptides covalently linked to their corresponding mRNA at the C terminus and DNA-modified amino-coated glass slides through hybridization of the nucleic acid components.[116] These addressable proteins could be visualized on the array both by autoradiography and by specific monoclonal antibody binding, and they were compatible for use in massively parallel formats. Lesaicherre et al. recently developed an intein-mediated protein-expression system to express, purify, and biotinylate proteins site-specifically so that immobilization onto avidin-functionalized glass slides could follow.[37]

As another system that uses fused proteins, the yeast two-hybrid system is one of the most extensive techniques for analyzing protein–protein interactions.[117–119] Generally, the method involves the generation of yeast "bait" proteins fused with transcription–activation DNA domains. The bait proteins can be probed with other yeast "prey" proteins fused with DNA-binding domains. This approach successfully identified 957 potential protein–protein interactions with 5300 arrayed yeast bait proteins and yeast prey proteins.[118]

In parallel with the yeast two-hybrid system, many exciting challenges have been undertaken to develop protein-detecting microarrays aimed at uncovering protein networks in biological systems. Zhu et al. generated a yeast proteome chip comprising recombinant proteins from 5800 open reading frames and identified many known calmodulin kinases and calcineurins in addition to 33 new binding partners of calmodulin with a potential binding motif.[32] Furthermore, Zhu et al. identified a total of 150 different proteins with phosphoinositide-binding activities against six different types of liposomes; they also revealed that 52 (35%) of the lipid-binding proteins corresponded to uncharacterized proteins and 45 proteins of the 98 known proteins were membrane-associated and either possess or were considered to have membrane-spanning regions. The fused-protein microarray approach is extremely attractive and will become main stream among the methodologies to carry out protein–protein interaction analysis in the microarray format as a result of the development of high-throughput protein purification and probe-attachment protocols. The affinity tags can also be employed as linkages to be attached with corresponding surfaces through site-specific/noncovalent interactions, thereby accelerating the development of the fused-protein microarray technology. Such an excellent strategy, however, still has some bottlenecks: 1) certain post-translational modifications may not be present in the recombinant proteins, depending on the expression system; 2) expression/purification of membrane proteins is a critical problem; and 3) assay protocols require antitarget antibodies modified with labeling group(s) or quantitative labeling techniques for proteins of interest in complex mixtures.

4.3. Phage-displayed antibody- and protein-fragment microarrays

Although many monoclonal antibodies that can bind to their specific antigens are now commercially available, the time-consuming and highly expensive processes involved in hybridoma technology are still a bottleneck. Phage display is one of the conventional and very powerful combinatorial biology methods. The technology follows the principle that polypeptides fused to bacteriophage coat proteins can be displayed on the surface of phage particles that also contain the encoding gene.[120,121] In this manner, a correlation between genotype and phenotype is established and extremely diverse libraries ($>10^{11}$) of DNA-encoded polypeptides or proteins can be produced and purified by molecular biology methods from *E. coli*.[122] Therefore, by providing alternative scaffolds for the capture agents, the methodology of phage-displayed artificial antibody fragments and/or polypeptides with alternative scaffolds has emerged. Fragments of antibodies, single-chain Fv fragments (scFv), and Fab fragments have been employed as capture agents (mostly scFv) on protein-detecting microarrays.

Fab fragments are heterodimers consisting of the antibody light chain and the V_H and C_{H1} domains of the heavy chain. scFv fragments are recombinant molecules consisting of a single polypeptide containing only the variable regions of the heavy and light chains joined by a flexible linker. Although scFv fragments are less stable and specific than Fab fragments and full antibodies due to the fact that they lack the V_H and C_{H1} domains, simpler scFv fragments are vigorously expressed on the phage surfaces to screen specific capture agents from a large-scale library.

Sheets et al. generated 6.7×10^9 members of phage-displayed human scFv fragments.[123] By using 14 different proteins for affinity selection, specific antibodies were successfully isolated with each antigen, and the average number of different scFv fragments generated per antigen was 8.7; for example, four different scFv recognizing the ErbB2 protein had affinities ranging from 220 pM to 4 nM. Gao et al. developed a phagemid format wherein antibody heavy- and light-chain variable regions were fused to the N termini of pVII and pIX, respectively.[124] The fused proteins interacted to form a functional Fv-binding domain on the phage surface to capture model proteins. Knappik et al. developed fully synthetic libraries having a human antibody framework with randomly integrated complementarity-determining region (CDR) cassettes, which have affinities ranging from 10^{-6} to 10^{-11} M against various antigens including haptens, DNA, peptides, and proteins.[125] Söderlind et al. constructed a scFv antibody library that permits human CDR gene fragments of any germ line to be incorporated combinatorially into the appropriate positions of the variable-region frameworks VH-DP47 and VL-DPL3, thereby affording some antibody fragments bound to haptens, peptides, carbohydrates, and proteins with dissociation constants in the subnanomolar range.[126] de Wildt et al. developed a technique for high-throughput screening of recombinant antibodies, involving robotic picking and high-density gridding of bacteria containing the antibody gene followed by filter-based ELISA to identify clones that express binding antibody fragments, thereby allowing up to 18342 different antibody clones to be screened at a time against bovine serum albumin (BSA), human serum albumin (HSA), and several recombinant human proteins.[127] In the near future, antibody fragments might compete with the following alternative scaffolds:[128,129] fibronectin,[130,131] lipocalin,[132] and ankyrin or leucine-rich repeat domains[133] although these systems still have not reached the maturity of phage-displayed antibody fragments.

Very recently, reviews have reported on the promising antibody phage-display technology involving the generation of antibody phage-display libraries[134] and the exploration of protein–protein interactions with such libraries.[135] Both reviews suggest that further study involving optimization and automation in the generation of phage-displayed proteins (polypeptides) are required to study a large number of different proteins in parallel.

4.4. DNA/RNA aptamer microarrays

Aptamers are oligonucleotide molecules (ten to several hundred building blocks) generated from SELEX process.[136] Aptamers have the potential characteristics of both proteins and nucleic acids. Aptamers can be easily synthesized and amplified and can compete with antibodies in affinity to targets including proteins.[137] Lee and Walt developed a fiber-optic biosensor by using an aptamer receptor for the measurement of thrombin, in which an antithrombin DNA aptamer was immobilized on the surface of silica microspheres and distributed in microwells on the distal tip of an imaging fiber.[138] The obtained fiber-optic microarray system showed a detection limit of 1 nM for nonlabeled thrombin and could be reused without any sensitivity change. Photoaptamers, a new class of aptamers that bear a photo-cross-linking functionality, were investigated. Smith et al. prepared an anti-HIV-gp120 photoaptamer modified with 5-bromo-2-deoxyuridine and immobilized it in a microarray format to detect subnanomolar concentrations of a target protein in 5% human serum.[139] The levels in sensitivity and specificity described by photoaptamers together with other advantageous properties of aptamers such as sophisticated synthesis and amplification protocols should facilitate the development of protein-detecting microarray technology.

Recently, Bock et al. reported photoaptamer arrays applied to multiplexed proteomic analysis.[140] The analytical procedure with photoaptamers is as follows: 1) a photoaptamer array is incubated with a mixture of proteins, 2) it is washed under nondenaturing conditions to remove nonspecifically bound proteins, 3) it is exposed to UV light to activate the photoaffinity functional group, 4) it is washed by using harsh, denaturing conditions to remove non-photo-cross-linked protein to improve signal-to-noise ratio, 5) it is analyzed by using a single reagent such as NHS-AlexaFluor555, which reacts with primary amines in the protein structure. Photoaptamers can be specifically selected and offer a scalable, reproducible detection of proteins of interest.

Bulyk et al. created double-stranded DNA (dsDNA) oligonucleotide arrays to perform highly parallel analysis of DNA–protein interactions.[141] They showed *dam* methylation of dsDNA arrays by digestion with *Dpn*I, which cleaves when its recognition site is methylated, a result indicating that this dsDNA array approach is applicable to explore the spectrum of sequence-specific protein binding sites in the proteomic study.

Aptamers, however, have some disadvantages; these are relatively lower stability in the presence of degradative enzymes in the biological samples and also lower diversity of binding features due to only 4 different components (A, T, G, C/U) compared with proteins comprising 20 different amino acids. Chemical modification of each component[142] would be useful to overcome these obstacles and generate highly stable, specific, and varying aptamers.

4.5. Peptide microarrays

Peptides, in general, have some superior features compared with proteins. They are 1) inexpensive, 2) highly stable against

dryness and oxidation, and 3) easy to manipulate, synthesize, and label with chromophores, but they sometimes lack high affinity and specificity against the target proteins. Over the past decade, peptide microarray technology has become a widespread and powerful tool to study molecular-recognition-mimicking protein–protein and antigen–antibody interactions and to identify biologically active peptides, despite the disadvantages discussed above. Applications such as epitope mapping and the characterization of protein–protein interactions, enzyme–substrate interactions, and inhibitory activity have been examined,[143] in accompaniment with the development of parallel peptide synthesis methodologies.[144,145] Indeed, in the applications of the peptide microarray, enzyme-profiling arrays to analyze enzymatic activities including inhibitory activities, especially phosphorylation catalyzed by protein kinases, have been frequently demonstrated.

MacBeath and Schreiber immobilized three different kinases substrates onto a glass surface.[31] A specific kinase activity together with isotope-labeled ATP was observed in the microarray format. Zhu et al. analyzed the activities of 119 protein kinases expressed and purified against 17 different substrates covalently attached to individual microwells by using isotope-labeled ATP.[33] Houseman et al. demonstrated quantitative evaluation of protein kinase activity by three different methods: 1) SPR spectroscopy, 2) fluorescence measurements, and 3) phosphorimaging with isotope-labeled ATP against substrate peptides immobilized covalently through Diels–Alder reaction of cyclopentene and benzoquinone moieties.[49] The SPR measurement was coupled with the anti-phosphotyrosine antibody, the fluorescence detection required the anti-phosphotyrosine antibody labeled with a fluorescent dye, and the phosphorimaging was performed by detecting the intensity of radioactivity from ^{32}P on the solid surfaces. Lizcano et al. provided the first description of the basis of the substrate specificity of NIMA (never in mitosis, gene A) related kinase-6 (NEK6) by isotope-labeled ATP on an aldehyde-modified surface.[146] Schutkowski et al. reported high-content peptide microarrays for revealing kinase specificity and biology, in which peptides attached with an aminooxyacetyl moiety as a reactive tag at the N terminus were immobilized onto the aldehyde-modified surface chemoselectively.[147] The same research group also analyzed the target specificity of Abl (proto-oncogene tyrosine-protein kinase ABL1) with a peptide microarray by using isotope-labeled ATP[148] and profiled CK2 (casein kinase 2) by using both radioactivity and anti-phosphopeptide antibodies.[149] Uttamchandani et al. reported a rapid method for the profiling of kinases by using a strategy that couples the merits of combinatorics (in rapid diversity generation) with the throughput attainable with microarrays (in parallel screening).[150] The peptides with an N-terminal cysteine residue were immobilized onto the surfaces by a thioester exchange reaction to form an amide bond and were detected by using fluorescently labeled anti-phosphoamino acid antibodies. In cases of arrays detecting enzymatic phosphorylation by kinases, isotope-labeled ATP or anti-phosphoamino acid antibodies are generally employed to monitor phosphorylation of each peptide substrate. Although detection of fluorescence intensity with an anti-phosphoamino acid antibody in the elucidation of protein kinase activities is a simpler and safer way to extract important information than the use of isotope-labeled ATP, the radioactivity approach is positively employed in the field so as to provide highly sensitive and reliable results exhibiting good discrimination between specific and nonspecific phosphorylated substrates.

Recently, Martin et al. demonstrated quantitative analysis of protein phosphorylation status and protein kinase activity on microarrays by using a novel fluorescent phosphorylation sensor dye, Pro-Q Diamond dye, instead of radioisotopes and anti-phosphoamino acid antibodies, and they found that characterization of enzymatic phosphorylation of immobilized peptides with the Pro-Q Diamond dye readily permits detection at picogram to subpicogram levels of sensitivity.[151] Ojida et al. also demonstrated fluorescence sensing of monophosphorylated peptides by bis(zinc(II)-dipicolylamine)-based artificial receptors.[152] Such chemically developable approaches that do not require any antitarget antibodies or isotope incorporation would be an alternative approach to the high-throughput screening method of kinase substrates.

Meanwhile, in arrays detecting protease activities, fluorescently labeled substrates are immobilized onto the surfaces, and when these substrates are processed, the resulting moieties emit more strongly to indicate enzymatic reactions at the defined positions in the array formats. Winssinger et al. demonstrated the detection of caspase activation upon induction of apoptosis and also performed characterization of the activated caspase. Inhibition of the caspase-executed apoptotic phenotype on the basis of enzymatic activities in crude cell lysates was also revealed by the use of a small-molecule-based (peptides and peptide mimics) profiling technique.[43] Salisbury et al. determined protease specificity by peptide microarrays, in which 7-amino-4-carbamoylmethyl coumarin (ACC) was used as a fluorogenic moiety for determining the P-site (N-terminal) substrate specificity of serine and cysteine proteases.[153] Yao and co-workers also showed that peptide substrates can be used for potential microarray-based screenings of activities from different classes of enzymes, including a protease, epoxide hydrolase, and phosphatase by using fluorogenic linkages on the surfaces;[154] this is discussed in more detail later. It is possible to obtain proteolytic "fingerprints" of proteases against combinatorial substrate libraries in a microarray format and to obtain kinetic parameters in each event of interest. Furthermore, if possibilities for detecting other classes of functional-group alterations derived from post-translational modifications, including proteolytic digestions and phosphorylations, were pursued, rapid progress would be made in protein-detecting technology. The same research group also developed a strategy for activity-based detection of enzymes in a protein microarray, in which enzymes were immobilized onto the surfaces and treated with mechanism-based inhibitors modified with a fluorescent dye.[155] As described above, these approaches, involving candidates of substrates and inhibitors based on the synthetic peptides, do not directly address a study of whole proteomes but rather are useful for detecting specific enzymes as biomarkers in diagnostics and

screening highly potent drugs in a miniaturized and parallel fashion.

Falsey et al. demonstrated the preparation and optimization of a novel type of microchip, which consists of glyoxylyl-modified glass microscope slides, and its application for high-throughput analysis of biomolecules and even whole cells.[156] Peptides (or small molecules) were spotted and covalently immobilized on the surfaces in a site-specific fashion through thiazolidine or oxime bond formation, and phosphorylation assays as well as whole-cell binding assays were carried out on the microarray. Very recently, Melnyk and co-workers constructed peptide microarrays, which were stable for a month and in which peptides were immobilized through semicarbazone linkages that are formed by coupling of glyoxylyl peptides and semicarbazide-modified slides.[55,56] The arrays detected specific antibodies by an immunoassay with three model epitopes (HCV core, NS4 capsid, and EBV capsid)[55] and with HCV peptide fragments, HBV (HBc, HBe, and HBs), HIV (gp41, gp120, and gp36), Epstein–Barr virus (VCAp18 153–176 peptide), and syphilis (rTpN47 and rTpN17) antigens.[56] The same group also constructed protein microarrays in which proteins were adsorbed onto the semicarbazide-modified surfaces, and they analyzed interactions with HIV (gp120, gp41), HCV (mix-HCV, core, NS3, and NS4), and HBV (HBs) recombinant antigens.[157] Meanwhile, immunoglobulin E (IgE) epitope mapping of food allergens is a prerequisite for engineering hypoallergenic immunotherapeutic agents and can provide important information regarding a patient's immune response. Schreffler et al. developed an immunoassay based on a peptide microarray to map peanut epitopes.[158] Although the analysis was successfully performed with good correlations to previous results, a diversity of patient IgE responses to the allergen was found. The epitope diversity obtained, however, should lead to improved therapeutic strategies and better monitoring of immunotherapeutic interventions. Again, the peptides are stable and easy to prepare, label with fluorescent dye(s), and equip with a synthetic handle for immobilization onto the surfaces. Although peptides are extensively applied in enzyme-substrate arrays to analyze enzymatic activities in the current situation, due to the convenience of this method for establishing a highly sensitive activity-based microarray, other applications of peptide microarrays have also been investigated in the field of detection of peptide–protein interactions.

Bialek et al. described the development of a process for the genome-wide mapping of interactions between protein domains and peptide ligands entirely based on high-throughput microarray technology.[159] It is possible to attach a phage library displaying protein domains from a randomly fragmented and cloned complementary DNA (cDNA) library onto a peptide microarray; peptide-specific phage populations are then automatically eluted, propagated, labeled, and identified by hybridization to a DNA microarray after multiple enrichment. Takahashi et al. constructed a novel protein-detection system in which 126 de novo peptides designed to form loop structures were labeled site-specifically with a fluorescent dye and immobilized into 96-well plastic plates.[52] In this system, when a protein of interest reacts with the peptide library, each peptide on the surface exhibits a characteristic increase in fluorescence intensity depending on a feature of the protein–peptide complex formation. Consequently, the peptide library comprising 126 peptides displays a unique fluorescent bar code corresponding to each protein of interest, called a "protein fingerprint". Usui et al. also demonstrated a protein-detection system comprising a de novo designed α-helical-peptide library in both liquid and solid formats to expand the diversity of use of the protein-fingerprint technique; each peptide in this system was equipped with two different fluorophores to allow a fluorescence resonance energy transfer (FRET) system.[53,160] The protein-fingerprint technique established above may allow profiling of a greater number of proteins with fingerprints than the number of capture agents immobilized on the chip, unlike protein-detecting microarrays in which antibodies and other proteins are immobilized. Moreover, the use of de novo designed peptide scaffolds labeled with fluorescent dye(s) does not require any fluorophore labeling of analytes. These kinds of arrays can be easily developed by a combination of organic chemistry and computational chemistry, and they can facilitate peptidomimetic drug discovery.

4.6. Molecularly imprinted polymer microarrays

Although, in principle, antibodies are very attractive capture agents for the design of protein-detecting microarrays to analyze protein abundances in a mixture sample, the poor chemical and physical stability of biomolecules probably prevents their broader use under biophysical conditions. Molecularly imprinted polymers (MIPs) are emerging as one of the alternative approaches and involve the use of biomimetic receptors capable of binding the analyte of interest with relatively high affinity and specificity.[161–163] Synthetic MIPs are formed by a process involving copolymerization of functional and cross-linking monomers in the presence of the analyte of interest as a template molecule. Subsequent removal of the template molecule reveals binding sites that are complementary in size and shape to the analyte.

The MIP approach has been developed to capture relatively small molecules. Senholdt et al. demonstrated determination of cyclosporin A and its metabolites in blood by using MIPs; the assay method comprised extraction of haemolyzed whole blood with organic solvent followed by a competitive radioimmunoassay of MIP particles and [^{3}H]-cyclosporin.[164] Turkewitsch et al. prepared MIPs against cyclic adenosine monophosphate (cAMP) that contained a fluorescent dye serving as both the recognition element and the measuring element for the fluorescence detection of cAMP in aqueous media.[165] This fluorescent MIP system displayed a quenching in fluorescence in the presence of cAMP with an association constant (K_a) of $\approx 10^5\ \text{M}^{-1}$, whereas almost no effect for the structurally similar cGMP.

Shi et al. reported a method for imprinting surfaces with protein-recognition sites.[166] They used plasma deposition to form polymeric thin films around proteins of interest coated with disaccharide molecules. The disaccharide molecules are covalently attached to the polymer film and create poly(sac-

charide)-like cavities that exhibit highly selective recognition for albumin, IgG, lysozyme, ribonuclease, and streptavidin. Klein et al. used a covalently modified tripeptide, Lys–Trp–Asp with 2-methacryloylbenzoyl chloride at α- and ε-amino terminus of the lysine, as a template.[167] After copolymerization of the template, the obtained MIPs were hydrolyzed with NaOH to remove the template and leave the carboxy group in the precise spatial arrangement for interaction with the target tripeptide. Lotierzo et al. synthesized an MIP film for domoic acid (DA) by direct photografting onto a gold chip suitable for an SPR-based bioanalytical instrument.[168] The obtained MIPs had approximately three times higher detection limits than those of monoclonal antibodies (5 ng mL^{-1} versus 1.8 ng mL^{-1} of DA, respectively). However, the detection range of the MIP sensor was considerably improved (5–100 ng mL^{-1}) compared to the immunosensor. As described above, the MIPs are stable, inexpensive, versatile, and easy to be prepared. The preparation of the MIPs and the examination of their binding abilities against target proteins can also be automated. Furthermore, the broad detection range obtained above might be due to the varied distribution of the polymer binding sites, which presented a variety of different affinities for the target molecule.[169] In the MIP approach, detection of relatively small molecules has been successfully demonstrated so far. In order to extend the usability of MIPs in proteomic studies, the construction of more homogenous binding sites for the target molecule is important in the synthetic polymers.

4.7. Carbohydrate microarrays

Carbohydrates in glycoproteins, glycolipids, and proteoglycans have important roles in biological systems, such as cell adhesion, migration, and signaling. Carbohydrate microarrays are an emerging technology to detect carbohydrate–protein interactions on miniaturized and parallel platforms.[170–173] Willats et al. immobilized structurally and chemically diverse glycan structures directly and stably onto the slide surfaces generated by treatment with black polystyrene followed by oxidation.[174] The obtained saccharide microarrays were highly reproducible and stable, and they could be stored in dry conditions for several months, thereby meeting the criteria required in use for protein-detecting microarrays. To analyze carbohydrate–protein interactions, Fukui et al. prepared oligosaccharide microarrays in which oligosaccharides derived from glycoproteins, proteoglycans, glycolipids, or synthetic oligosaccharides were displayed on a nitrocellulose membrane.[175] The oligosaccharide microarrays revealed that carbohydrate-recognizing proteins, interferon-γ and the chemokine RANTES interacted not only with oligosaccharides of chondroitin sulfates but also with sulfated sequences, such as the HNK-1 sequence characteristic of natural killer cells and the Lewis^a and Lewis^x series which are known to occur on epithelial cells.

In order to extend the scope of biomedical research on carbohydrate-mediated molecular recognition such as antiinfection responses, Wang et al. immobilized 48 microbial polysaccharides onto a nitrocellulose membrane and incubated the system with human sera to detect human serum antibodies.[176] This microarray system allowed repertoires of human antibodies with anticarbohydrate binding activities to be probed and a wide range of microbial infections to be characterized. In addition, identification of a broad spectrum of IgG isotypes of human anticarbohydrate antibodies indicated that the isotype profile in normal individuals may differ from those in the repertoires of human myeloma and lymphoma cells. Very recently, Adams et al. developed carbohydrate and glycoprotein microarrays to analyze glycan-dependent HIV-gp120–protein interactions.[177] The binding profiles of five HIV-gp120 binding proteins and the carbohydrate structural requirements for these interactions were also determined. This may offer a potential strategy for HIV-vaccine development. The carbohydrate microarrays described above exhibit high stability, sensitivity, and the potential to analyze and discover known and/or new carbohydrate-mediated molecular recognition in a parallel fashion. This technology will allow focusing on the carbohydrate-mediated events on a proteome-wide scale without interference by any other abundant proteins, in what is called a "focused proteome study."

4.8. Small-molecule microarrays

In parallel with the development of antibody arrays and fused-protein arrays, small-molecule microarrays have also been developed. MacBeath et al. created microarrays of thiol-containing small molecules, through a Michael addition based immobilization reaction, and used the microarrays to measure 10 800 binding events involving three different proteins in a single experiment on a single glass slide.[178] Schreiber and co-workers also developed the immobilization methodology of alcohol-containing small molecules onto thionyl chloride activated glass slides and analyzed interactions with three different proteins in a highly efficient and selective manner.[179] Other small-molecule-based microarrays were used in a parallel manner to screen 3780 1,3-dioxane derivatives with a fluorescently labeled yeast protein, Ure2p, that represses the transcription factors Gln3p and Nil1p, thereby affording the result that one compound activated a glucose-sensitive transcriptional pathway downstream of Ure2p.[180] The same group also expanded small-molecule microarrays by demonstrating immobilization of small molecules with a phenol, carboxylic acid, or sulfonamide functionality that has an acidic proton (and therefore cannot be attached covalently onto the chlorinated slides described above) onto diazobenzylidene-functionalized glass slides.[181]

Zhu et al. developed a microarray-based strategy for the detection of three major classes of hydrolytic enzymes, that is protease, epoxide hydrolase, and phosphatase, on the basis of their catalytic activities; in this system, their substrates which comprise two different units, a fluorogenic moiety and an enzyme-recognition head, including coumarin-conjugated epoxide, phosphate, and positively/negatively charged amino acids, were immobilized onto the surfaces.[154] When the enzyme recognition head is cleaved by proteases, the fluorogenic moieties (7-amino-4-carbamoyl coumarin and 7-hydroxymethyl coumarin) left on the surfaces emit much more strong-

ly, thereby indicating the existence of the target enzyme in the sample solution.

Very recently, Baldini et al. reported a simplified approach based on the use of solution arrays of fluorescent-protein surface receptors, in which a model small library of tetraphenyl porphyrin derivatives functionalized with different amino acids or amino acid derivatives is placed in the wells.[182] Titration of the protein into the porphyrin solution resulted in quenching of the porphyrin fluorescence due to complex formation, and the response of the array corresponds to a unique fingerprint, characteristic of a specific protein. These studies are related to the work done by Mihara and co-workers, who have demonstrated such protein-surface exploration with synthetic peptide libraries tethering fluorescent dye(s) and who have shown the potential of using characteristic protein fingerprints in profiling proteins.[52,53,160] Small-molecule microarrays are stable and easy to prepare, and they have the potential to accelerate target-protein confirmation, including enzyme and ligand discovery.

4.9. Cell and tissue microarrays

Analyses of gene and protein expression levels by DNA microarrays and protein-detecting microarrays, respectively, are just beginning to provide important information about the biological functions of bioorganisms. Cell and tissue microarrays would enable the sample amounts required to be reduced and would accelerate the processes of biological evaluation of ligands to discover potent drugs and biomarkers. Some cell[183] and tissue[184] microarrays have already been created and studied with gene activity, protein expression, cell-surface exploration, and so on. These technologies are beyond the scope of this review but should undoubtedly be powerful diagnostic and drug discovery tools.

5. Summary and Outlook

There is increasing interest in the importance of protein-detecting microarray technologies in proteomic studies. In the coming few years, the protein-detecting microarray will become a significantly promising and indispensable research/diagnostic tool, as discussed above. In this review, we have highlighted the features of three basic technologies (surface chemistry, detection methods, and capture agents) that are independent but strongly related to each other and the current accomplishments and requirements in order to understand the situation of surrounding microarray technology and to share the bright prospects for this technology in proteomic studies. The important factors in developing protein-detecting microarray technology are summarized below:

1) Since a variety of surface-modification methodologies are available, it is necessary to prepare microarray platforms, including soft membranes and polymer-coated and chemically modified solid supports, that can offer highly sensitive, reproducible, and inexpensive analyses appropriate to every target molecule.
2) Fluorescence-detection systems exhibit potential for highly sensitive and parallel analysis to provide a large amount of important information with a reduced volume of sample solution within a single experiment. Improvement in reproducibility and quantification is desired, for example, by combining in parallel the method with other potential analytical methods. The SPR imaging approach that is still under development would be helpful for precise and reliable analyses in proteomic studies.
3) Antibodies, their fragments, DNA/RNA aptamers, and MIPs will probably be settled on as the capture agents for analyzing protein abundances in complex protein mixtures. Fused-proteins, phage-displayed protein fragments, peptides, and other classes of small molecules are promising candidates as the capture agents for revealing protein functions. In particular, peptides and small organic molecules are potentially applicable to facilitate the processes in drug discovery and target confirmation. Such capture agents are still on the way to being developed and must be diversified to meet the requirement of ideal protein-detecting microarray technology.

Great efforts have been devoted so far to addressing the fabrication and application of protein-detecting microarrays, and this technology has begun to offer frameworks both for comprehensive and focused proteomic studies in the next generation. Indeed, a comprehensive yeast-proteome microarray has been released commercially in 2004.[185,186] In the near future, it is possible that protein-detecting microarrays will be the body of biological and diagnostic tools available for analyzing protein abundances under various conditions and for studying protein–protein interactions that connect protein networks in complex cellular events. We are now still at the middle point in the establishment of an ultimate proteome-analyzing system that will help us to understand the secrets of life more broadly and deeply.

Keywords: analytical methods · arrays · capture agents · proteins · surface chemistry

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