

Review

Protein and peptide arrays: Recent trends and new directions

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Abstract

Microarrays of proteins and peptides make it possible the screening of thousands of binding events in a parallel and high throughput fashion; therefore they are emerging as a powerful tool for proteomics and clinical assays. The complex nature of Proteome, the wide dynamic range of protein concentration in real samples and the critical role of immobilized protein orientation must be taken into account to maximize the utility of protein microarrays. Immobilization strategy and designing of an ideal local chemical environment on the solid surface are both essential for the success of a protein microarray experiment. This review article will focus on protein and peptide arrays highlighting their technical challenges and presenting new directions by means of a set of selected recent applications.

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Contents

1. Introduction	77
2. Types of protein microarrays	78
3. Surface chemistry and architecture	79
3.1. Surface chemistry	79
3.2. Molecular architecture of the surface	80
4. Capture ligand	81
4.1. Antibody/antigen microarrays	81
4.2. Peptides as capture ligand	81
5. Detection	82
5.1. Label-free methods	82
5.2. Labelled probe methods	83
5.2.1. Fluorescence	84
5.2.2. Chromogenic detection	84
5.2.3. Chemiluminescence	85
5.2.4. Radioactivity	85
6. Non-planar microarrays	85
7. Concluding remarks	85
Acknowledgement	85
References	85

1. Introduction

In recent years, microarrays have become an invaluable research tool for life scientists and their use in diagnostics has emerged as a great promise of medicine. Microarrays consist of immobilized biomolecules spatially addressed on substrates

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such as planar surfaces (typically coated microscope glass slides), microwells or arrays of beads. Immobilized biomolecules, here referred as probes, usually include oligonucleotides, PCR products, proteins, peptides, carbohydrates and other small molecules. Ideally, probes should retain activity, remain stable and not desorb during the experimental steps. The collection of molecules arranged (in arrays) on the substrate is then probed with cellular extracts, serum, PCR products or other samples chasing for molecular recognition events. Minimal non-specific binding of biomolecules to the surface is therefore one of the most important criteria for high quality microarray experiments.

Microarrays have rapidly attracted the interest of users, due to their high throughput since the appearance of two seminal papers by MacBeath and Schreiber (2000) and Zhu et al. (2001). In spite of the problems related to the manufacture and use of microarray technology for proteins (Predki, 2004), a variety of interesting applications of this technique have appeared in the literature in the last years as reported by several recent reviews (Venkatasubbarao, 2004; Tomizaki et al., 2005; Angenendt, 2005; Templin et al., 2003; Merkel et al., 2005). This review article, without pretending of being exhaustive, will focus on protein and peptide arrays highlighting some of their technical challenges and the new trends by means of a set of selected recent applications.

2. Types of protein microarrays

A general scheme of a typical protein array experiment is provided in Fig. 1: a large set of capture ligands (proteins or peptides) is arrayed on a solid support, after washing and blocking surface unreacted sites, the array is probed with a sample containing (among a variety of unrelated molecules) the counterparts of the molecular recognition events under study. If an interaction occurs, a signal is revealed on the

surface (by a variety of detection techniques). By scanning the entire array a large number of binding events are detected in parallel.

Protein arrays generally fall into three categories:

- (1) function arrays;
- (2) detection arrays (or analytical arrays);
- (3) reverse phase arrays (Zhu and Snyder, 2003).

In protein function arrays (which are generally aimed at discovering protein function in fundamental research) a large set of purified proteins or peptides or even an entire proteome is spotted and immobilized. The array is then used for parallel screening of a range of biochemical interactions. Protein function arrays (Blackburn and Hart, 2005) can be used to study the effect of substrates or inhibitors on enzyme activities (Zhu et al., 2003; Dietrich et al., 2004) protein-drug or hormone-effector interactions (Kim et al., 2005; Lee et al., 2004) or in epitope mapping studies (Shreffler et al., 2005; Poetz et al., 2005; Bialek et al., 2003; Chiari et al., 2005).

In protein detection microarrays, an array of affinity reagents (antigens or antibodies) rather than the native proteins themselves, is immobilized on a support and used to determine protein abundances in a complex matrix such as serum (Combaret et al., 2005; Hamelinck et al., 2005; Sanchez-Carbayo et al., 2006). Analytical arrays can be used to assay antibodies (for diagnosis of allergy (Hueber et al., 2005; Bacarese-Hamilton et al., 2005) or autoimmunity diseases (Robinson et al., 2002a,b) or to monitor protein expression on a large scale (Wingren and Borrebaeck, 2004; Kopf et al., 2005). In a third category of protein arrays (usually referred as reverse phase microarrays), tissues (Speer et al., 2005), cell lysates (Geho et al., 2005) or serum samples (Janzi et al., 2005) are spotted on the surface and probed with one antibody per analyte for a multiplex readout (cf. Section 5.2).

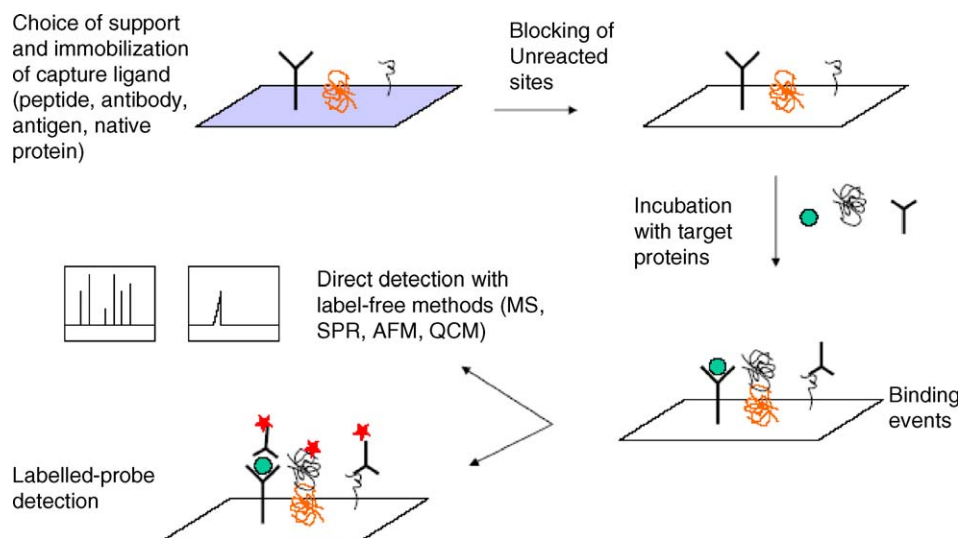


Fig. 1. General scheme of a typical protein microarray experiment. A set of capture ligands (proteins, antibodies, peptides) is arrayed onto an appropriate solid support. After blocking surface unreacted sites the array is probed by incubation with a sample containing the target molecules. If a molecular recognition event occurs, a signal is revealed either by direct detection or by a labelled probe. MS: mass spectrometry, SPR: surface plasmon resonance, AFM: atomic force microscopy, QCM: quartz crystal microbalance.

3. Surface chemistry and architecture

The aim of peptide and protein microarrays is the study of molecular interactions occurring between two partners: one contained in a liquid sample and one immobilized on a solid support. The surfaces typically used for immobilization of DNA are often not suitable for proteins owing to the biophysical differences between the two classes of bioanalytes (Kusnezow and Hoheisel, 2003).

The key requirements of the surface hosting a protein microarray assay are:

- (1) Provision of an optimal binding capacity of capture ligands (probes).
- (2) Retaining of biological activity of capture ligand (proteins tend to unfold when immobilized onto a support, in order to allow internal hydrophobic side chains to form hydrophobic bonds with the solid surface (Butler, 2000)).
- (3) Accessibility of the ligand by the interaction partner (protein–substrate interactions reduce the accessibility of the target, possibly leading to false negative results). This issue is particularly important for peptide microarrays due to the small molecular mass of capture ligands.
- (4) Low degree of aspecific interaction (the achievement of a low degree of aspecific binding is extremely difficult when the sample is a complex mixture of thousands of molecules such as serum (Kusnezow and Hoheisel, 2003)).

3.1. Surface chemistry

A general classification of ligand attachment mechanisms is given in Fig. 2.

The simplest way for binding a protein is through surface adsorption. This approach has been used in standard ELISA and Western blot for many years and is based on adsorption of the macromolecules either by electrostatic forces on charged surfaces (poly-lysine coated slides (Haab et al., 2001)) or by hydrophobic interactions (nitrocellulose or polyvinylidene fluoride (PVDF) membranes (Bussow et al., 1998)). Nitrocellulose coated slides exhibit an excellent binding capacity

and a long term stability of the printed probes. However, non-specific binding to nitrocellulose can be a significant problem. In spite of its simplicity, the adsorption method presents several drawbacks:

- (i) the attached proteins can be removed by stringent washing conditions;
- (ii) the background level is usually high due to non-specific protein adsorption–desorption;
- (iii) proteins adsorbed on hydrophobic surfaces tend to denature (Kusnezow and Hoheisel, 2003).

The covalent binding of proteins and peptides on the substrate represents a more robust approach.

Considerable effort has been made in the last years to improve immobilization of proteins on modified glass surfaces using a number of different strategies which recently have been compared systematically (Guillaume et al., 2005). The covalent mechanism of attachment requires the presence of reactive groups on the support (usually electrophilic groups such as epoxides (Zhu et al., 2000), aldehydes (MacBeath and Schreiber, 2000), succinimidyl esters/isothiocyanate functionalities (Benters et al., 2001)) able to react with nucleophilic groups (amino, thiol, hydroxyl) on the ligand molecules. The functional groups on the surface are introduced by glass modification with organosilanes such as 3-glycidypropyltrimethoxysilane (GOPS) or 3-aminopropyltriethoxysilane (APTES). Alternatively they can be inserted on more complex molecular architectures such as self-assembled monolayers (SAM) (Schaeferling et al., 2002) or polymers grafted to the surface. Organosilanes can directly provide the functional groups for ligand attachment (GOPS) or react with a bifunctional ligand bearing the desired reactive group. A microarray surface has been developed (Lee et al., 2003a,b) with ProLinkerTM, a calixcrown derivative with a bifunctional coupling property that permits efficient immobilization of capture proteins on solid matrices such as gold films or aminated glass slides.

The use of a matrix that embeds the protein in a structured environment is an alternative way to immobilize proteins. This mechanism does not involve the cross linking of the capture molecules with the surface but is based on the physical entrapment of proteins in gels such as polyacrylamide (Arenkov et al., 2000) or agarose (Afanassiev et al., 2000). The three-dimensional structure of these substrates generally increases the loading capacity and does not disturb the potential functional sites or regulatory domains of the protein; moreover, the aqueous environment of the gel reduces protein denaturation. However, the gel structure can represent a barrier to the diffusion and the molecular recognition events may require long incubation times. A mixed approach, based on nanoengineered 3D polyelectrolyte thin films (PET) deposited on glass slide by consecutive adsorption of polyelectrolytes via self-assembly technique, has been reported (Zhou and Zhou, 2006). Proteins were immobilized in the PET-coated glass slides by electrostatic adsorption and entrapment on porous structure of the 3D polymer film.

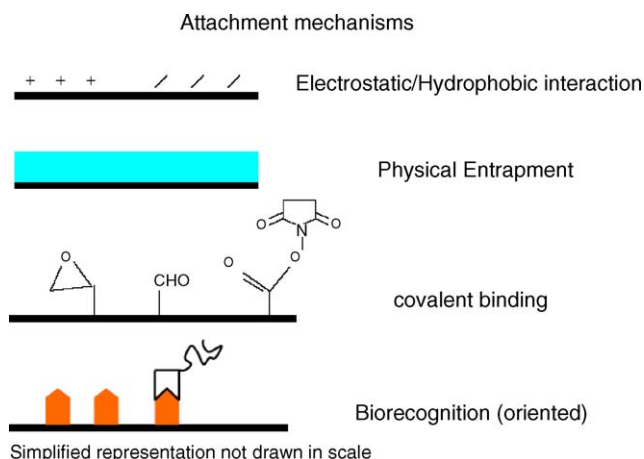


Fig. 2. Simplified representation of the most common attachment mechanisms for capture ligands.

The protein microarrays on the PET films provided a dynamic ranges up to three orders of magnitude wider than the same protein microarrays fabricated on conventional aldehyde and poly-L-lysine functionalized slides.

Whether the attachment occurs by adsorption, physical entrapment or by a covalent binding, the immobilization takes place in a random or non-specific orientation.

In addition to non-specific methods, probe molecules can be tagged and immobilized by a specific non-covalent interaction between the tag and an immobilized capturing molecules. The attachment to the surface is typically mediated by a molecular recognition event such as biotin–avidin interaction, by interaction between nickel coated slides and His-tagged proteins or glutathione and GST tags (Zhu et al., 2001). An innovative affinity-based protein immobilization strategy was developed based on the combined use of the carbohydrate binding module (CBM) and its natural three-dimensional substrate such as cellulose (Ofir et al., 2005). CBM fused antibodies or peptides were produced, immobilized on cellulose surfaces and used for serodiagnosis of human immunodeficiency virus patients.

Using affinity-based strategies, the immobilization occurs in an oriented, uniform and specific mode. The disadvantage of this approach relies on the fact that proteins must be biotinylated or tagged. In a recent article, Cha et al. (2005) compared the catalytic activities of enzymes immobilized on silicon surfaces with and without orientation; the authors established the crucial role of the probe orientation for a reliable use of protein microarrays as quantitative tools in biomedical research.

3.2. Molecular architecture of the surface

Besides the attachment mechanism (adsorption, physical entrapment into gels, covalent binding or oriented molecular recognition), also the molecular architecture of the coating plays an essential role and must be considered. A schematic representation of common surface molecular architectures is provided in Fig. 3. Proteins or peptides, regardless of the attachment mechanism, can be directly in contact with the activated glass surface on a one-dimensional coating;

alternatively, the probe can be inserted in a simple monolayer architecture such as in SAM or poly ethylen glycol (PEG) (Jun et al., 2004) or in a more complex three-dimensional structure like a dendrimeric polymer (Benters et al., 2001) or a random co-polymer (Cretich et al., 2004). The use of a co-polymer of *N,N*-dimethylacrylamide, *N*-acryloyloxysuccinimide, and 3-(trimethoxysilyl)propyl methacrylate, copoly(DMA-NAS-MAPS), to covalently attach biomolecules on glass slides was firstly reported for the preparation of low-density DNA microarrays (Pirri et al., 2004). The innovative aspect in this approach relies in the fact that the polymer self-adsorbs onto the glass surface very quickly, simply immersing glass slides in a diluted aqueous solution of the polymer and without time consuming glass pretreatments. Therefore, the coating procedure is a fast and inexpensive method of producing hydrophilic functional surfaces bearing active esters, able to react with amino groups in modified DNA, proteins and peptides.

The copoly(DMA-NAS-MAPS) slide performance was investigated in the assessment of rheumatoid factor (RF) in human serum samples (Cretich et al., 2004). The results demonstrate that the immobilized proteins maintain an active conformation and are easily accessible; moreover, after the assay, the slides exhibited a very low background. The polymeric surface was also tested as a peptide microarray support in an epitope mapping study (Chiari et al., 2005). This study suggested that although the copoly(DMA-NAS-MAPS) slides bind the capture molecule in a random conformation, the aqueous micro-environment created by the polymeric coating provided a good accessibility of the ligand without need for a spacer between the probe and the surface.

Very recently a new method to develop polymeric microarray surfaces has been introduced (Dai et al., 2006) based on coating of substrates with polyelectrolyte multilayers terminated with poly(acrylic acid) (PAA) followed by activation of the free –COOH groups of PAA. This surface both resist non-specific adsorption and allow for covalent immobilization of arrays of active antibodies. Moreover, deposition of these coatings on microporous alumina supports resulted in a 500-fold increase in surface area relative to two-dimensional supports, leading to decreased protein-microarray detection limits by 2 orders of magnitude.

Supramolecular structures are generally considered favorable in retaining protein activity. In addition, due to the decreased reaction rate constant in close vicinity to the surface, recognition of small molecules, such as peptides, is often hindered. The polymeric coating on the surface acts as a linker and moves the bound probe away from the surface resulting in a much faster and efficient reaction with the target. This issue is very important when dealing with small molecule microarrays as peptide arrays (cf. Section 4.2).

Besides suitable surfaces, printing buffers must be developed to provide stable and physiological conditions to the proteins spotted on the surface (e.g. glycerol, sucrose, saccharose are often added to PBS which is the most common printing buffer). Solubility is often an issue for hydrophobic peptides suggesting the use of detergents in the spotting buffer.

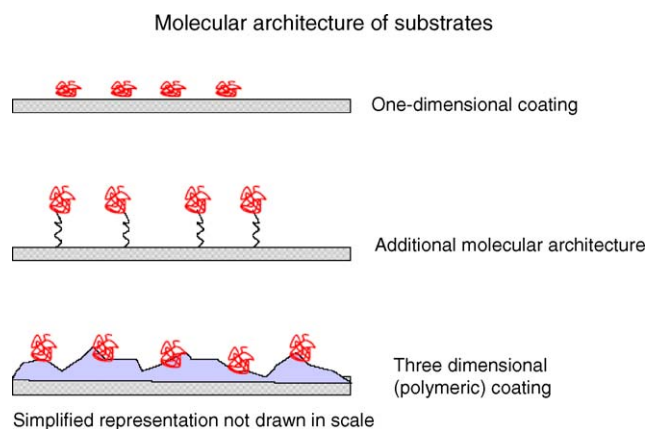


Fig. 3. Simplified representation of the most common surface molecular architectures.

Despite the lack of an ideal universal surface or immobilization approach, as demonstrated by the literature, it is clear that existing methods are more than adequate for many applications (Templin et al., 2003) and in general, the functionality of immobilized proteins appears to be robust to a wide variety of immobilization strategies.

4. Capture ligand

In contrast to DNA microarrays, for which the capture probes are easily designed and synthesized, the selection and production of the capture agents are the most critical points in protein-detecting microarrays (Tomizaki et al., 2005). A protein capture agent must be able to recognize its target among 10,000 of other protein species in a sample and then retain it throughout the assay (Phelan and Nock, 2003). Therefore, the capture ligand must be highly specific for the protein of interest and with an affinity sufficient to capture even proteins at very low concentration. The other important characteristic of a protein capture agent is its stability. This issue is highly critical for the ability to retain activity during immobilization on the surface and the various steps of the assay which can require stringent conditions for removing non-specific interactions. There is a wide variety of capture ligands in use in protein microarrays. The major class of these are antibodies due to their high stability, specificity and affinity to target molecules. Antigens, peptides and non-protein-based molecules such as DNA (Collett et al., 2005; Winssinger et al., 2002), RNA (Lee et al., 2005) or PNA (Winssinger et al., 2004) are used as well.

4.1. Antibody/antigen microarrays

The most critical requirement for antibody microarray realization is the establishment of methods for selection, production and purification of antibodies with high affinity and reduced cross reactivity. The development process of high quality antibodies is usually expensive and labour intensive. For monoclonal antibodies (mAb), once cell hybridomas have been created, the clones will provide potentially limitless antibodies with single-epitope specificity (Goding, 1996). The production of polyclonal antibodies is limited in quantity by the amount of serum that can be obtained by the immunized animal. Polyclonal antibodies contain multiple epitope specificity but can sometimes provide greater specificity than mAb if the undesired cross reactivity is removed by immunoaffinity purification using purified antigens (Phelan and Nock, 2003).

One of the most important goals in protein-detecting microarrays is to analyze protein abundance change in biological samples such as biopsies or serum in a very large dynamic range using a two colour approach. Antibody microarrays containing more than 200 polyclonal and monoclonal antibodies specific for cell cycle, apoptosis and nuclear signalling proteins are commercially available. They have been recently used for the analysis of protein expression profiles of F9 embryonic carcinoma cells by a two colour approach (Kopf et al., 2005).

The cross reactivity of monoclonal and polyclonal antibodies against 5000 yeast proteins has been tested (Michaud et al., 2003). The authors found out that the antibodies not only recognized cognate proteins but also cross-reacted with a number of unrelated yeast proteins at various degree. These results suggest the use of an alternative procedure such as ELISA or immunoblotting tests to validate the antibody microarray outcomings. Poetz et al. (2005) have recently introduced a miniaturized and parallel microarray system consisting of proteins and peptides that defines the epitope recognized by a given binder with the potential to speed up the screening process for the generation of recombinant antibodies with pre-defined selection criteria.

In diagnostic applications the use of the antigen–antibody immunoresponse is particularly effective. Robinson et al. (2002a,b) developed autoantigen arrays for the specific detection of autoantibodies in serum from patients with eight autoimmune diseases. Antigen microarrays are widely used for allergy diagnostics for determination and monitoring allergic patients' IgE reactivity profiles (Hiller et al., 2002). In the last decade, advances in molecular allergology made it possible to clone and produce the allergens of the major allergenic sources (pollens, mites, foods, moulds, venoms) in recombinant forms. These proteins are available in large scale quantities, retain their immunological properties and can be used as capture ligands to detect serum IgE. (Jahn-Schmid et al., 2003). The use of antigen microarrays is not restricted to IgE mediated diseases. Mezzasoma et al. (2002) have developed an antigen microarray for the serodiagnosis of infectious diseases based on IgG and IgM detection directed against several microbial and viral antigens. Recently, Steller et al. realized a protein microarray with 67 proteins of the known phase-variable genes from *Neisseria meningitidis* responsible for invasive diseases. The protein array was screened with healthy controls and affected patient sera revealing 47 immunogenic proteins that can be used as disease markers and that have diagnostic and medical potential (Steller et al., 2005).

4.2. Peptides as capture ligand

Synthetic peptides have some very interesting features as capture ligands: they can mimic biological activities of proteins, they are easy to synthesize and manipulate, they are usually highly stable and inexpensive. Unfortunately they sometimes lack high affinity and specificity against target proteins. Since peptides have small molecular mass they are not easily accessible when non-specifically adsorbed on solid supports (Seong and Choi, 2003). Moreover, since peptides often lack a well-defined three-dimensional structure, a correct orientation is essential to promote their interaction with the target (Lesaicherre et al., 2002). For these reasons, immobilization of short peptides often requires covalent linkage of the compounds onto a solid support. Chemoselective N-terminal attachment of peptides on slides have been developed based on glyoxylic acid and N-terminal cysteine-containing peptides (Falsey et al., 2001), on Diels Alder reactions (Houseman et al.,

2002), on native chemical ligation and on avidin–biotin interaction (Falsey et al., 2001). In a covalent immobilization of a molecule, the site of ligation is often important, especially for molecules containing multiple biological active sites. For example, if a peptide requires its free amino and sulfidryl groups to be active, it is not possible to use standard aldehyde or *N*-hydroxyl succinimide chemistries for its attachment (Xu and Lam, 2003). In some applications, the immobilization of the ligand onto a glass surface via a long hydrophilic linker (or by proteins) may be beneficial for its biological activity as demonstrated by Falsey et al. (2001) in the phosphorylation of a peptide substrate by the p60c-src protein tyrosine kinase. The properties of the support can be critical to the performance of the peptide array. Supports that do not promote non-specific adsorption, for example, would reduce the level of false positive results and ensure that a higher fraction of the immobilized peptide is available for interaction with the targets. The most common strategy to prevent false positive interactions is to treat the array with a blocking protein such as BSA that adsorb to the surface to prevent further unwanted adsorption in the assay. However, BSA can also block the interaction of immobilized peptides (Min and Mrksich, 2004), therefore a covalent attachment followed by blocking with a non-protein quenching agent such as triethanolamine is often preferred when using peptide microarrays. Another important issue with peptide microarrays is the uniformity of peptides density in each spot that is essential to enable direct comparison of relative activities across the array.

A different approach in the development of peptide arrays is the SPOT-synthesis developed by Frank (2002) based on the parallel synthesis of peptides directly on cellulose membranes. The major drawback of this approach, (and of the photolithographic in situ light mediated synthesis) relies on the fact that the same peptides must be synthesized many times at different location whereas in the spotting approach a lot of spots of the same peptide can be produced by the same peptide solution synthesizing the compound only once. Despite the difficulties in handling small molecule arrays, microarraying peptides has demonstrated to be a very promising strategy for epitope mapping (Chiari et al., 2005), detection of pathogen infections (Duburcq et al., 2004) or signalling-dependent changes of molecular interactions (Stoevesandt et al., 2005). Enzyme-profiling arrays to analyze enzymatic activities, especially phosphorylation catalyzed by protein kinases, has been frequently reported (Uttamchandani et al., 2004). Peptide arrays have also been used to identify ligands that are active in cell adhesion (Falsey et al., 2001) and, in another interesting application aimed to develop peptidic inhibitors of β -lactamases, using a phage display library (Huang et al., 2003). Peptide arrays have been a topic of interest for many years but the utility of this tool in a diverse set of applications has been demonstrated only in the past years (Min and Mrksich, 2004).

5. Detection

Current detection strategies for protein microarrays are generally classified as (Espina et al., 2004):

- (1) Label-free methods, including mass spectrometry (MS), surface plasmon resonance (SPR) and grating-coupled surface plasmon resonance (GC-SPR), atomic force microscopy (AFM), micro-electromechanical systems (MEMS) cantilevers and quartz-crystal microbalance analysis (QCM).
- (2) Labelled probe methods, including fluorescence, chemiluminescence, electrochemiluminescence and radioactivity detection.

5.1. Label-free methods

Protein microarrays based on Surface-enhanced laser desorption/ionization (SELDI) TOF MS, employ an “on chip” selection of the proteins of interest from a complex mixtures and subsequent ionization of the retained molecules to a detector for a classification based on the mass/charge ratio (Bischoff and Luider, 2004).

SPR spectroscopy is a well-know technique to study the kinetics of protein–protein interactions in real-time (Johnsson et al., 1991) and it's the leading technology in the field of label-free detection of molecular interactions (Ramachandran et al., 2005). In conventional SPR spectroscopy, the capture ligand is immobilized on a thin metal film (gold or silver) and the target is added. The change in the reflection angle of the incident light indicates the amount of target molecule captured on the surface. SPR is a versatile tool but it allows the analysis of only a few channels for a single experiment. Recently, up to 400 real-time antibody-target bindings could be measured simultaneously within a single hour using antibody microarrays combined with SPR technology (Usui-Aoki et al., 2005).

Grating-coupled surface plasmon resonance (GC-SPR) is a method for the assessment of analyte in a multiplexed format. In GC-SPR, the analyte is flowed across specific probes (e.g. antibodies) that have been immobilized on a sensor chip. The chip surface is illuminated with p-polarized light that couples to the gold surface's electrons to form a surface plasmon. At a specific angle of incidence, called the GC-SPR angle, the maximum amount of coupling occurs, thus reducing the intensity of reflected light. Since regions of the chip can be independently analyzed, this system can assess 400 interactions between analyte and receptor on a single chip (Unfricht et al., 2005).

AFM reveals the change in height of an immobilized protein upon binding with its cognate molecule. It has been introduced for detection at the singular molecular level in protein nanoarrays generated by Dip-Pen Nanolithography (Lee et al., 2002) but it is also currently widely used for surface characterization in protein microarrays (Lee et al., 2003a,b).

Microcantilevers for biosensing are silicon strips attached at one end, with the capture molecule (antibody or protein) bound to the surface. When an analyte binds onto the microcantilever, its bending as a result of surface stress is measured by the deflection of an optical beam or by a change of electrical resistance in a piezoelectric thin film on the cantilever. Alternatively, a change in its mechanical resonant frequency is measured. Recently, a label-free detection of a prostate-specific

antigen (PSA) with a detection sensitivity as low as 10 pg/mL has been demonstrated using a nanomechanical cantilever (Lee et al., 2005). The authors of this work detected PSA via electrical measurement of the resonant frequency change generated by the molecular interaction of the antigen and the antibody. The resonant frequency shifted due to the specific binding of the PSA to its antibody which is immobilized via calixcrown self-assembled monolayers on an Au surface deposited on a nanomechanical cantilever.

QCM is a sensitive mass-measuring system at the nanogram level (Matsuno et al., 2001) which can be used to monitor biological events such as molecular interactions in real-time.

Carbon nanowires and nanotubes, though at an early stage, have the potential to address the needs of label-free sensing in protein arrays (Ramachandran et al., 2005). They consist in a wire with a mean diameter of 30–100 nm and lengths of 5–10 nm. The sensing system is based on a change in conductance as proteins bind to a functionalized nanowires connecting two electrodes. The multiplexed electrical detection of cancer markers using silicon-nanowire was demonstrated by Zheng et al. (2005). In this work protein markers were detected at femtomolar concentrations with high selectivity. Very recently, protein recognition via Surface Molecularly Imprinted Polymer Nanowires was proposed. These imprinted surface nanowires exhibit highly selective recognition for a variety of template proteins, including albumin, hemoglobin, and cytochrome *c* (Li et al., 2006).

All label-free detection methods are promising tools to characterize binding events on surfaces. They do not require labelling of molecules that may affect protein activity. However, they are generally based on sophisticated equipment not easily available in all clinical laboratories.

Coupling protein microarrays to real-time and label-free detection systems compatible with high-throughput methods would strongly enhance the ability to understand protein function on a proteome scale.

5.2. Labelled probe methods

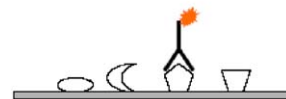
Labelled probe methods have directly evolved from clinical immunoassays, radioimmunoassay or ELISA protocols where they are widely used. They can be distinguished in (see Fig. 4):

- (1) Direct (when a mixture of proteins is immobilized and the detection is performed using labelled binding molecules such as antibodies).
- (2) Indirect (when immobilized antibodies are used as capture ligands and probed by labelled proteins).
- (3) Sandwich (when a first immobilized antibody acts as a capture agent for the assayed protein which is revealed by a recognition with a secondary labelled antibody).

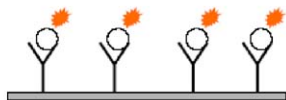
In the direct approach, usually referred as reverse phase protein microarray (Liotta et al., 2003) each spot of the array contains an individual test sample. Therefore, an array may comprise several sera of different patients or cellular lysates containing a complex mixture of proteins. The array is then

Detection Methods using Labelled-Probes

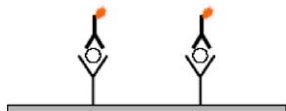
1: Direct (specific detection with labelled antibody)



2: Indirect (capture antibodies are probed with labelled proteins)



3: Sandwich assay with labelled secondary antibody



Simplified representation not drawn in scale

Fig. 4. Simplified representation of the labelled probe methods commonly used in protein microarray detection.

incubated with one detection protein (typically an antibody) allowing the comparison of a single analyte in different samples.

In the indirect detection approach, a known capture ligand is immobilized on the surface and probed by a labelled complex mixture of proteins. The test sample can be a cellular lysate or a serum in which multiple analytes are measured simultaneously. In a two colour approach, pharmacological treatments or protein expression profiles can be compared (Kopf et al., 2005).

The sandwich assay format rely on immobilized antibodies for capturing the protein of interest while a second labelled antibody directed against the captured protein is used for detection. In this approach, two distinct antibodies, each with affinity to separate epitopes on the protein of interest are required.

The bottlenecks of labelled-probe methods are the production of antibodies and the quantitative labelling of antibodies/antigen. Existing collections of analyte specific antibodies cover a limited fraction of the proteome. The Human Protein Atlas Initiative (<http://www.proteinatlas.org>) part of Human Antibody Initiative by Human Proteome Organization (HUPO) represents an important effort in the direction of generating a catalogue of validated antibodies.

Labelled probe detection methods rely on fluorescence, chromogenic, chemiluminescence, electrochemiluminescence or radioactive labelling strategies. Detection methods developed for microarrays, due to the miniaturized format, are required to provide high sensitivity (high signal to noise ratio) and high throughput. The use of fluorescent probes and signal amplification techniques with chromogenic or fluorescent probes usually leads to performances that meet such criteria.

The Rolling Circle Amplification (RCA) has been developed to improve sensitivity in fluorescence detection and was first applied to protein microarrays in 2000 (Schweitzer et al., 2000) for the detection of antigens down to the femtomolar level. RCA signal amplification system relies on the use of a “reporter” antibody, conjugated to an oligonucleotide. The reporter Ab binds to the test analyte captured on the microarray solid surface. A DNA circle hybridizes to a complementary

sequence in the oligonucleotide. The resulting complex is washed to remove excess reagents, and the DNA tag is amplified by DNA polymerase. The amplified product is labelled *in situ* by hybridization with fluorescently labelled oligonucleotides. After amplification, RCA products remain localized at the microarray spot and the sensitivity improvement is typically of 1000-fold (Kingsmore and Patel, 2003). RCA has been recently combined to nanoparticle-based optical detection (Hsu and Huang, 2004). The use of light-scattering particles as fluorescent analogs in biological and clinical applications was first demonstrated in 1998 (Yguerabide and Yguerabide, 1998). Nanogold-based optical detection can provide a highly sensitive detection procedure easier than fluorescence techniques. Moreover, the stability of the samples is improved leading to the possibility of reanalyzing the samples.

5.2.1. Fluorescence

Fluorophores exist in different formulations with narrow emission and excitation spectra, they are becoming more and more popular and are nowadays the method of choice for microarray detection (Angenendt, 2005). Well-established protein labelling methods rely on fluorescein (Finnskog et al., 2004), cyanines (Wiese, 2003), rhodamine (Houseman et al., 2002), acridines, phycobiliproteins, and Bodipy (Coleman et al., 2004) compounds. The choice of a fluorescent probe depends on the sample type, emission spectra and substrate. For example, the sample itself may have an autofluorescence interfering with the fluorophores like in the case of flavoproteins that emit light in the same region of fluorescence. Recombinant proteins with red and green fluorescent tags were used to investigate protein–protein interactions (Kukar et al., 2002).

The Cy3 and Cy5 dyes are widely used for fluorescent detection of proteins. These dyes are widely used in DNA microarrays due to their reduced photobleaching and quenching. The two colour detection approach with Cy5 and Cy3 that has been successfully applied for comparing protein levels of two different samples. Multicolour detection schemes are especially beneficial as microarray measurements are not quantified absolutely but relatively. Simultaneous detection of the species permits a direct comparison of the fluorescent intensity in different samples without interchip variations. In the assay the two differently labelled samples compete for the binding with the same immobilized antibody target.

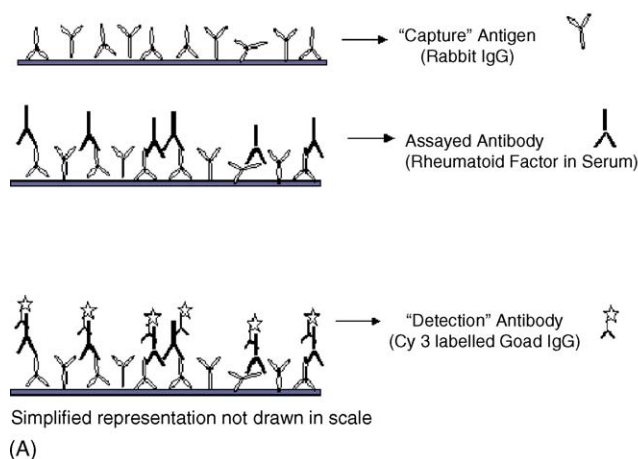
The fluorescence sensing system coupled with antibodies represents the simplest and most versatile strategy for an highly specific and sensitive analysis (Tomizaki et al., 2005). Even though the requirement of two antibodies severely constrains the array design, the sandwich method, increases the specificity of antibody arrays and is widely used especially in microimmunoassays on array format. The assessment of Rheumatoid Factor (RF) in human serum samples in a sandwich microimmunoassay was recently demonstrated on a polymeric glass coating (Cretich et al., 2004). The scheme of the sandwich assay is reported in Fig. 5A. The assay provided reproducible results and a linear dose–response relationship

with a detection limit of 900 amol per spot as shown in Fig. 5B which reports the results of a typical microarray experiment.

5.2.2. Chromogenic detection

Chromogens are substrates for an enzymatic reaction that generates an insoluble coloured product. Commonly used enzymes for chromogenic reactions on microarrays are alkaline phosphatase and horseradish peroxidase. These enzymes act on colourless chemical substrates generating stable coloured products. For example the blue spots generated by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) and alkaline phosphatase system has been used for

Scheme of Sandwich Microimmunoassay of Rheumatoid Factor (RF)



Results of Rheumatoid Factor (RF) assay

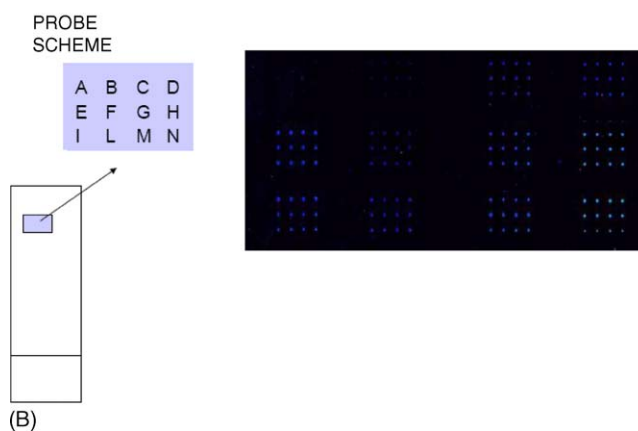


Fig. 5. (A) Schematic view of the sandwich assay. The capture antigens, immunoglobulins from rabbit serum (rIgG), were spotted on the slides. After overnight binding, slides were washed and hybridized with human serum samples containing different amounts of the assayed antibody (RF). RF was revealed by recognition with a secondary antibody, the anti-human goat immunoglobulins (Goat-IgG) labelled with Cy3. (B) Portion of protein microarray obtained at the end of the sandwich assay described in (A). The spotted capture antigens were spotted according to the scheme. A: BSA (negative control) 1 mg/mL, B: rabbit IgG 0.2 mg/mL, C: rabbit IgG 0.4 mg/mL, D: rabbit IgG 0.5 mg/mL, E: rabbit IgG 0.6 mg/mL, F: rabbit IgG 1.0 mg/mL, G: rabbit IgG 1.2 mg/mL, H: rabbit IgG 1.4 mg/mL, I: rabbit IgG 1.6 mg/mL, L: rabbit IgG 1.8 mg/mL, M: rabbit IgG 2.0 mg/mL, N: rabbit IgG 2.2 mg/mL. The microarray was incubated with a human serum sample containing 183.6 IU/mL RF.

proteomic profiling of cancer cells on nitrocellulose coated slides (Knezevic et al., 2001). Tetramethylbenzidine (TMB) and diaminobenzidine (DAB) are widely used substrate for peroxidase. This detection system provided a femtomolar sensitivity in microarray assays (Paweletz et al., 2001).

5.2.3. Chemiluminescence

Chemiluminescence, which is the luminescence generated by a chemical reaction, is generally used in microarrays for the detection of proteins recognized by a secondary antibodies labelled with alkaline phosphatase or horseradish peroxidase. The enzymatic oxidation of substrates such as luminol produces a prolonged emission of light which is captured by a charge-coupled device (CCD) camera. Chemiluminescence, although very sensitive has a limited dynamic range (Schweitzer et al., 2003).

5.2.4. Radioactivity

Radioactive labelling is mainly performed using different isotopes such as ^{32}P or ^3H incorporated in proteins or by a ^{125}I triiodothyronine probes. Signal detection is visualized by autoradiography and quantified by a densitometer (Weng et al., 2002; Falsey et al., 2001; Ge, 2000). Radioactivity is traditionally one of the most sensitive labelling procedure. The technique uses fully active proteins and allow to precisely quantify the amount of protein assayed. Due to safety concerns and waste issues, it's not widely used anymore. Moreover it's not fully compatible with high throughput screening methodologies.

6. Non-planar microarrays

Conventional techniques for patterning biomolecules on planar substrates normally involve direct application of spots on surfaces using highly automatized microdeposition instruments. The planar format is widely used nonetheless the typical planar array sometimes suffers from non-uniformity and slow diffusion of targets to the binding surface (Zhi et al., 2003). Moreover, several authors have demonstrated that an active mixing of hybridization solution results in a gain of sensitivity and improves accuracy and reproducibility of microarrays results (Bynum and Gordon, 2004; Adey et al., 2002; Schaupp et al., 2005). A different approach to overcome this problem consists on the use of encoded microparticle arrays to perform hybridization in a “solution-like” environment. Unlike positional or spatial encoding, in which it is the exact location on the array surface that allows the identification of the analyzed molecule, in non-planar arrays, each microcarrier has to be encoded to assess the identity of each probe. The microcarriers with different probes attached to the surface can be mixed in the same vial that contains the target analyte. In this solution-like environment, the reactions proceeds with solution kinetic. The unique code on each microcarrier allows either the ligand or the compound attached to the carrier surface to be unequivocally identified. The various strategies developed for microcarrier encoding have been reviewed in depth (Braeckmans et al., 2002). Spectrometric, graphical, physical and electronic encoding systems have been developed.

Another advantage of the use of microparticles such as latex or glass beads in array format assays concerns the increased binding surface provided by tridimensional particles (Marquette et al., 2003).

7. Concluding remarks

Protein microarray technology is not as straightforward as DNA technology due to the complex nature of proteins that can be hydrophobic or hydrophilic, acidic or basic. Posttranslational modifications further enhance protein molecular variability. Moreover the wide dynamic range of protein concentration in real samples presents great challenges for detection strategies (Zhu and Snyder, 2003; Espina et al., 2004).

A challenging aspect in protein microarray technology development is the difficulty in maintaining the native state of the protein upon surface immobilization. Moreover, as many proteins are labile, the overall process in a microarray experiment (expression, purification, spotting and microarray storage) must be planned and carried out in order to maintain protein integrity (Ramachandran et al., 2005). A useful strategy in this regard has been proposed by Ramachandran in 2004 (Ramachandran et al., 2004). The authors of this work spotted protein expression plasmids instead of purified proteins on the microarray surface generating a nucleic acid programmable protein array (NAPPA) which reduces the process of building protein microarray to a single step.

The steadily increasing amount of literature on protein microarrays, have demonstrated an even larger variety of protein activities in microarray formats (Cha et al., 2005). Nonetheless, the critical role of immobilized protein orientation and the designing of an ideal local chemical environment on the solid surface are both essential if protein microarray technology is to be used as a quantitative tool in biomedical applications (Cha et al., 2005). The technical concerns on the design of the optimal microarray surface together with improved and straightforward detection methods are the key to the success of microarray experiments (Guillaume et al., 2005) and must be addressed to maximize the usefulness of protein microarrays in the large-scale study of protein interactions.

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