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Review

Solid supports for microarray immunoassays

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Stimulated by the achievements of the first phase in genomics and the resulting need of assigning functions to the acquired sequence information, novel formats of immunoassays are being developed for high-throughput multi-analyte studies. In principle, they are similar in nature to the microarray assays already established at the level of nucleic acids. However, the biochemical diversity and the sheer number of proteins are such that an equivalent analysis is much more complex and thus difficult to accomplish. The wide range of protein concentration complicates matters further. Performing microarray immunoassays already represents a challenge at the level of preparing a working chip surface. Arrays have been produced on filter supports, in microtiter plate wells and on glass slides, the last two usually coated with one-, two- or three-dimensionally structured surface modifications. The usefulness and suitability of all these support media for the construction and application of antibody microarrays are reviewed in this manuscript in terms of the different kinds of immunoassay and the various detection procedures. Additionally, the employment of microarrays containing alternative sensor molecules is discussed in this context. The sensitivity of microspot immunoassays predicted by the current analyte theory is not yet a reality, indicating the extent of both the technology's potential and the size of the task still ahead.

Keywords: **protein microarray; immunoassay; antibody; protein interaction; expression profiling; surface chemistry**

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FROM DNA-MICROARRAYS TO MICROSPOT IMMUNOASSAYS

DNA-microarrays have become an essential tool in the functional interpretation of sequence information obtained from the various genome projects. Although indispensable for the understanding of global variations at the level of nucleic acids, they provide only limited insight into the process of actual protein expression and even less information on protein–protein interaction or the proteins' biochemical activity. Many aspects of modulation and regulation of cellular activity cannot be investigated at the level of nucleic acids, since they occur by post-transcriptional control of protein translation (McCarthy, 1998), post-translational modifications (Parekh and Rohloff, 1997) or protein degradation by proteolysis (Marcotte, 2001; Kettman *et al.*, 2002). The situation is further complicated by the fact that no function is known for a large percentage of the predicted proteins of multicellular organisms (Edwards *et al.*, 2000). Also, the dynamic range of protein expression ranges widely and could be as large as seven orders of magnitude (Pandey and Mann, 2000; Kettman *et al.*, 2002). For the purpose of really understanding cellular functioning at the protein level, the interest in new, large-scale technologies in the field of proteomics has grown enormously. A good measure for this is the number of new journals dedicated to proteome research (Laurell and Marko-Varga, 2002) as well as the growing awareness of the inherent commercial potential (CHIreports, 2003).

Antibody/antigen microarrays represent a methodology that is compatible with DNA microarrays, since it aims at a simultaneous analysis of several thousand proteins of biological samples. However, while some microspot immunoassays like antibody mini-arrays (Moody *et al.*, 2001) and antigen microarrays (Robinson *et al.*, 2002) have been successful in demonstrating the usefulness of the technology in principle, their performance has only been moderately productive. At current, antibody microarray systems are basically incapable of producing data from small samples, which are frequent in a clinical environment (Knezevic *et al.*, 2001). However, even with more material, many arrayed antibodies still produce very weak or no signal (Sreekumar *et al.*, 2001). Only analyses of samples of low complexity were really successful (Schweitzer *et al.*, 2002). Comprehensive studies are still some distance away and will require a multidisciplinary effort for their establishment (James, 2002). In order to advance antibody microarrays from an elegant concept to a reliable tool in proteomics,

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Abbreviations used: PEG, polyethylene glycol; PLL-g-PEG, poly-L-lysine grafted PEG copolymers; PSA, prostrate-specific antigen; SAM, self-assembled monolayer.

many technical hurdles in the areas of surface chemistry, large-scale production of specific binders and antigen labelling and detection will have to be overcome. Surface chemistry is one of the initial key issues (Kusnezow and Hoheisel, 2002). In the following, we will mainly focus on the topics relevant to the performance of solid supports for antibody/antigen microarrays.

SURFACE PARAMETERS

The report of MacBeath and Schreiber (2000) on protein microarray production and analysis by means of standard arraying equipment and slide scanners available from DNA microarray studies opened new perspectives in functional and comparative proteomics. However, the chemical aspects of DNA microarray surfaces could not be adopted as easily as the hardware could, since there are fundamental biophysical and biochemical differences between the two classes of biological substances:

- proteins are chemically and structurally much more complex and heterogeneous than nucleic acids;
- in contrast to DNA, proteins easily lose their structure and biochemical activity due to denaturation, dehydration or oxidation;
- the detection of proteins by antibody–antigen interactions is characterized by a broad range of specificity and affinity. Additionally, binding affinities of antibodies were found to be reduced upon immobilization (Vijayendran and Leckband, 2001).

Consequently, there is a need for more sophisticated immobilization chemistries. Standard surfaces applied in ELISA- or radio-immunoassays cannot be adopted without reservations due to the basic characteristics of microarray immunoassays.

Background

Antibody microarrays create a new situation for immunoassay development, because all analytes in a directly labelled sample or all detectable analytes in sandwich assays produce a signal by non-specific binding. There is more background in a microspot-ELISA as the amount and total number of secondary antibodies that are used increases. In contrast to the equally negatively charged nucleic acids, proteins are amphiphatic molecules, which consequently exhibit pronounced surface activity (Hlady and Buijs, 1996). The high degree of protein adsorption is caused by electrostatic, van der Waals and Lewis acid–base forces, hydrophobic interactions as well as conformational changes and restricted lateral diffusion in the vicinity of a surface. The extent and kind of interaction with a surface differs widely between proteins. Therefore, achieving a low degree of unspecific binding is extremely complicated, if a complex protein sample containing thousands of molecules is to be analysed.

Complexity and sensitivity

Whereas the detection limit of DNA-microarrays enables the detection by fluorescence of high, medium and, in part, even low abundant transcripts (Kane *et al.*, 2000), much better sensitivity is called for by the comparably large spectrum of proteins as well as the wider range of concentrations present within a given sample. Despite the fact that the theoretical detection limit of a microspot array with antibody monolayers was predicted to be a few femtograms or less (Ekins, 1998), it has been difficult until now to produce a detectable signal in the low picogram range even with artificial one-antibody one-antigen test systems, which avoid the background problems described above.

Antigen–surface interaction

While the antibodies used in microarray experiments are similarly structured, globular proteins (IgG subtypes are mostly used for immunoassays), variations in size, structure and charge of antigens are enormous. The influence of this on array performance is often underestimated. Frequently, different surface chemistries are compared with a single antigen–antibody system (e.g. Joos *et al.*, 2000; Madoz-Gurpide *et al.*, 2001; Angenendt *et al.*, 2002). This does not take into account the effects of variations in the antigens. Variation in size, for example, strongly influences performance parameters on different surfaces (Kusnezow *et al.*, 2003). An obvious explanation is steric obstruction of the binding. However, specific surface activities or conformational changes upon immobilization may also contribute to this effect (Butler, 2000). Consequently, testing of microarray surfaces should be done with as many antigen–antibody pairs as possible.

Spot uniformity

On microarrays, the sensor molecules are presented in features that are formed concomitantly to the actual attachment process. Good spot quality in turn facilitates the subsequent evaluation of array data and has immediate effects on the reliability of the results. In our experience, hydrophobic surfaces tend to produce small but inhomogeneous spots, whereas most hydrophilic surfaces yield homogeneous spots, which, however, are often of irregular shape. The nature of the spotting solution also plays a considerable role. It should be emphasized that scarcely any one surface or attachment strategy will satisfy all the demands of all types of protein microarray experiments. Also, one might be forced to accept compromises for the reasons of cost and complexity of surface production and protein attachment.

ANTIBODY ATTACHMENT

In view of the chemical properties of antibodies (Fig. 1), there are several elementary processes that could be used to attach them to a surface. Physical adsorption and binding via amino groups of lysines and arginines are common to all protein classes. Strategies that are rather specific to antibodies include binding by way of their thiol groups, the most reactive of them being positioned either in the hinge region or between light and heavy chains (Horejsi *et al.*, 1997; Zhang and Czupryn, 2002), attachment via carbohydrate residues of the Fc region and coupling with proteins A or G (Table 1).

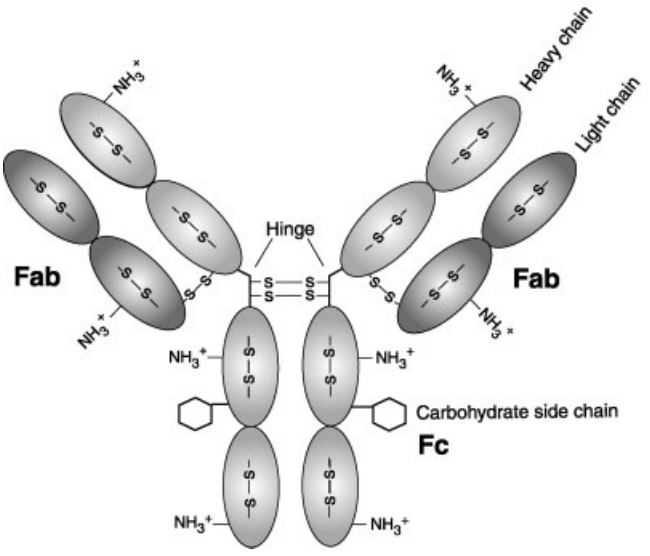

Figure 1. Schematic representation of an antibody structure. Reactive sites relevant to microarray immobilization are indicated.

Physical adsorption represents the simplest process of protein binding, although it is rather uncontrollable. Close proximity between the adsorptive surface and the protein's reactive site could influence the affinity for a ligand unfavourably. Surfaces may also be susceptible to exchanging adsorbed protein with proteins in the surrounding solution (Ball *et al.*, 1994; Lutanie *et al.*, 1992). Stringent washing can destabilize protein attachment. Proteins adsorbed to hydrophobic surfaces tend to denature (Metzger *et al.*, 2002). The binding mechanism on classical support media used for ELISA, such as polystyrene for example, is based on a partial denaturation of proteins on the hydrophobic surface, which may cause a substantial decrease in immunoreactivity (Butler, 2000). Adsorption on hydrophilic surfaces seems favourable in this respect. Antibodies spotted onto nitrocellulose-coated slides, for instance, could be stored over many months.

Covalent binding of proteins to a surface represents a more rational and robust approach and can be subdivided in random and oriented attachment. Overall, all oriented immobilization processes share several advantages. Usually, the active sites are better accessible to the analytes when the attachment site is positioned elsewhere. Within each antibody population, there is less variation in antibody affinities upon covalent immobilization and this affects sensitivity positively (Vijayendran and Leckband, 2001). Finally, one would expect a superior stability of the antibodies when there is a single attachment point (Wilchek and Miron, 2003).

Nevertheless, these positive effects arising from oriented attachment depend strongly on the actual surfaces and detection system applied. Anderson and colleagues compared protein A-coated slides vs mercaptosilane/maleimido-*N*-hydroxysuccinimide ester surfaces (Anderson *et al.*, 1997). While signal intensities were similar on both surfaces for directly fluorescently labelled samples, sandwich assays demonstrated a significantly better performance on protein A surfaces. Oriented immobilisation via the antibody carbohydrate groups (Nisnevitch *et al.*, 2000; Nisnevitch and Firer, 2001) was found to vary in performance dependent on the actual surface. No significant differences in performance parameters were observed for the best-performing random (mercaptosilane/heterobifunctional succinimide crosslinkers) and oriented (mercaptosilane/heterobifunctional hydrazide crosslinkers) binding processes (Shriver-Lake *et al.*, 1997; Kusnezow *et al.*, 2003). Peluso *et al.* (2003) also compared the performance of directed and random attachment of antibodies, which had been biotinylated via carbohydrate and amino groups. On surfaces with a very long spacer between a streptavidin-coated solid support, the antibodies that had been biotinylated at carbohydrate groups exhibited an up to 10 times higher signal intensity. Interestingly, the difference on surfaces with shorter linkers

Table 1. Summary of antibody immobilization strategies suitable for microarray production; for more information on bioconjugate chemistry see Hermanson (1996)

Antibody attachment method	Examples of surface derivatization	Mechanism of binding	Coupling procedure
Adsorption	Nitrocellulose, poly-L-lysine, aminosilane, polystyrene	Electrostatic and hydrophobic forces, etc.	Incubation
Covalent, random	Succinimidyl esters (NHS), epoxy-, aldehyde-, carbodiimide-containing compounds	Reactive primary amines of lysines and arginines	Incubation
Covalent, oriented via hinge region	Maleimides, disulphide derivatives, arylating agents	Mostly by cysteines of disulfide bridges in hinge region, but also cysteines at other sites	Reduction of disulphide bridges by cysteamine or mercaptoethanol
Covalent, oriented via carbohydrate residues	Amino groups, hydrazines	Carbohydrate residues of Fc portion of antibody	Oxidation of carbohydrates by sodium metaperiodate
Specific binding, oriented via proteins A, G or L	Proteins A, G or L	Affinity binding of Fc portion	Incubation; often subsequent treatment with bifunctional reagent
Introduction of specific functional groups, e.g. biotinylation	Suitable functions, e.g. streptavidine	Strong and specific interaction of functional groups	Incubation

was much lower. Comparison of two Fab-fragments biotinylated either randomly or at the thiol groups in the hinge region displayed a similar effect. Specificity of oriented binding over random attachment was 1.8- to 5.8-fold higher on surfaces with a short linker and 5- to 10-fold higher on surfaces with a long linker. Also, specific immunoglobulin binding activity of protein A could be significantly improved using oriented attachment of recombinant protein A in the first immobilization step (Kanno *et al.*, 2000).

However, directed attachment does not necessarily guarantee better results in all cases (Wilchek and Miron, 2003; Kusnezow *et al.*, 2003). In addition, from a practical point of view, immobilization of antibodies by thiol groups or carbohydrate is rather difficult to perform in microarray experiments due to the requirement of modifying the native antibodies prior to spotting. Loss of antibody activity has also been observed (Shriver-Lake *et al.*, 1997; Nisnevitch *et al.*, 2000). In addition, antibodies were lost during subsequent purification and concentration steps (Shriver-Lake *et al.*, 1997; Kusnezow *et al.*, 2003). Third, it is impossible to keep the antibodies in an active state for a long time. The production of microarrays, however, requires the handling of large numbers of expensive and sometimes rather unique protein molecules in small volumes. A complex attachment process including an antibody pre-treatment step is therefore hard to manage and may cause a loss of valuable resources.

Furthermore, attachment of the antibodies in a directed manner also has other disadvantages. Although oriented molecule layers result in a kinetically more homogeneous behaviour of the antibodies, this does not correlate with higher specificity. Frequently, a lower density of attached proteins is observed (Vijayendran and Leckband, 2001) since immobilization efficiency depends on the number of available coupling groups (Oates *et al.*, 1998). In addition, attachment by means of protein A or G can be applied to certain immunoglobulin subtypes only, which are bound by these proteins with high affinity (Anderson *et al.*, 1997; Turkova, 1999). Chemical reduction of the disulfide bridges in antibodies with subsequent immobilization on thiol-reactive surfaces (Rowe *et al.*, 1999; Karyakin *et al.*, 2000), another classical direct immobilization procedure, may suffer from non-uniform antibody binding. Susceptibility of inter-heavy (hinge region) and heavy-light chain disulphide bridges to reducing agents varies between different immunoglobulin subtypes (Bloom *et al.*, 1997). Consequently, binding by thiol groups may result in partial loss of antibody activity. Interestingly, however, mono- and polyclonal antibodies exhibited on maleimid surfaces relatively high signal intensities even without the additional step of antibody reduction (Kusnezov *et al.*, unpublished). This is probably due to the existence of free thiol groups, which was reported for most antibody classes (Horejsi *et al.*, 1997; Schauenstein *et al.*, 1982; Zhang and Czupryn, 2002).

FILTER ARRAYS

Filter membranes represent the support medium with the highest protein binding capacity. Low-density arrays were used successfully for the investigation of specific interactions of proteins with radioactive-labelled nucleic acids and other ligands (Ge, 2000) and the detection of cytokines in patient sera and cell culture media (Huang, 2001; Huang *et al.*, 2001). In the latter experiment, the detection limit on the antibody arrays was a few picograms of cytokine per millilitre. Bussow *et al.* (1998) produced a high-density antigen array made from a human fetal brain cDNA expression library of 37 830 clones for the purpose of antibody screening. Detection threshold by this approach was about 10 pg protein. A limitation of all filter arrays is the comparatively low resolution, the considerable background signal and the difficulties in automating the analysis process. Owing to the relatively large reaction volume required, it is also impracticable to use them in applications for which only limited sample quantities are available, such as protein expression profiling of tumour biopsies (Huang *et al.*, 2001c).

MICROTITRE PLATE FORMAT

This hybrid format originates from the demand for miniaturizing immunological assays performed in classical 96-well microtitre-plates, while still relying on this well-established platform itself (Emili and Cagney, 2000; Walter *et al.*, 2000; Laurell and Marko-Varga, 2002). Mini-arrays of up to 250 spots were printed onto the bottom of a well (Eggers *et al.*, 1994 (High Throughput Genomics, Inc., Tucson, USA); Mendoza *et al.*, 1999). While most such microspot immunoassays were performed on only few antibodies per well (Moody *et al.*, 2001; Wiese *et al.*, 2001; Tam *et al.*, 2002), there are commercial products with up to 50 antibodies (Eggers *et al.*, 1994). The main problem of this approach lies with the chemiluminescence usually used for detection. When a protein of high concentration binds to particular a spot, the signal may overlap with others produced at spots close by (Moody *et al.*, 2001). However, the attainable sensitivity and dynamic range of detection on these microarrays are considerable in comparison to conventional ELISA assays. Mini-arrays of less than 10 elements were used to screen cytokines in different biological samples with a sensitivity of a few pg/ml (Huang *et al.*, 2001a; Moody *et al.*, 2001; Wiese *et al.*, 2001; Tam *et al.*, 2002). Besides the conventional polystyrene surface (Moody *et al.*, 2001), micro-wells were coated with cyanosilane in several studies (Huang *et al.*, 2001a; Wiese *et al.*, 2001; Tam *et al.*, 2002). By this means, the antibodies were bound electrostatically via the glycosyl rests of their Fc regions (Falipou *et al.*, 1999).

Apart from the large volume required, the main disadvantage of the microtitre plate format is the inherent limitation with regard to further assay miniaturisation and thus volume reduction. Noteworthy advantages, however, are the capability of stirring the incubation solution—avoiding depletion effects—and the ability to process in parallel various protein samples. Combining the principle of separate wells with the format of microchips may well be a way to advance the technology and could be facilitate low complexity analyses on many different samples (Schweitzer *et al.*, 2002).

MICROCHIP FORMATS

Microarrays enable high-throughput analyte detection, allow for small sample volumes and are compatible with

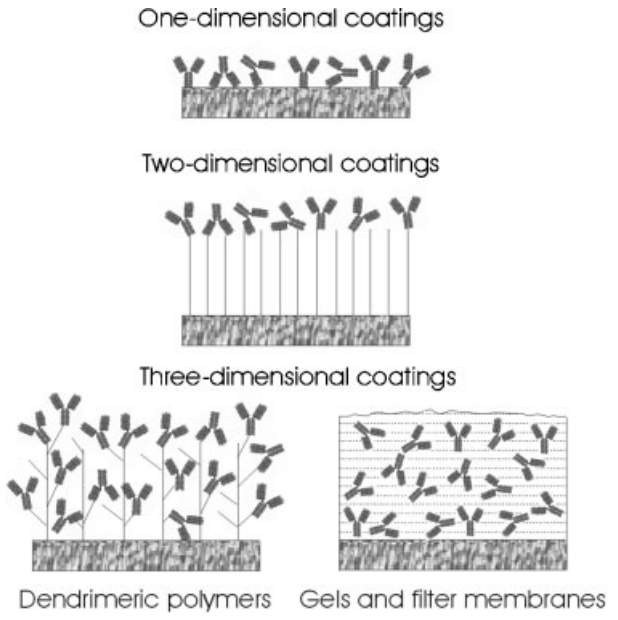

Figure 2. Graphical representation of various forms of slide coatings.

standard microarray equipment already in use for analyses at the level of nucleic acids. Additionally, glass support offers a large variety of different immobilization chemistries. In the following, we discuss microarray assays subdivided according to the structure of the linkage molecules used for the attachment of the antibody probes (Fig. 2).

One-dimensional coatings

Modifications of microarray glass surfaces with coatings such as poly-L-lysine or silanization with amino or aldehyde silane are typically used for the attachment of nucleic acids. Haab *et al.* (2001) developed antibody/antigen microarrays on poly-L-lysine-coated glass slides using fluorescent labelling of antigens or antibodies, respectively. Of 115 tested antibody/antigen pairs, only 50% of the antigens and 20% of the arrayed antibodies provided specific and accurate results. Antibody/antigen interactions could be detected with a dynamic range of three orders of magnitude and a sensitivity of about 1 ng/ml. Using the same technology, an autoantigen microarray of 1152 features was produced (Robinson *et al.*, 2002). It contained 196 distinct molecules (proteins and peptides) for the purpose of detecting antibodies in the serum of patients with autoimmune rheumatic disease. Applying a fluorescent sandwich assay, a much higher sensitivity was achieved compared with standard ELISA assays. However, the serum samples had been filtered beforehand and were significantly enriched in antibodies by this process, indicating that the complexity of serum samples was relatively low. Sreekumar *et al.* (2001) spotted 146 antibodies on poly-L-lysine and aldehyde slides. Co-analysing samples labelled with Cy3- and Cy5-dye, respectively, they aimed at the detection of protein variations in colon carcinoma cell lines that had been treated with ionizing radiation. Unfortunately, only data obtained on 20 of the 146 antibodies were published. The performance parameters shown in this report were comparable to the results above.

Alternatively to the techniques originating from DNA-microarrays, some classical antibody immobilization strategies were used for the construction of antibody microarrays. Schweitzer *et al.* (2002) produced a microarray of 51 cytokine antibodies, which were printed on thiolsilane-coated surfaces that had been activated with a bifunctional crosslinker. This kind of attachment is common in immunosensor production (Bhatia *et al.*, 1989; Shriver-Lake *et al.*, 1997). The reported sensitivity was about 1 ng/ml antigen using dual fluorescent labelling. Applying rolling circle amplification, cytokine secretion by dendritic cells in cell culture media which had been depleted of fetal calf serum could be detected in a semi-quantitative manner with a sensitivity of a few pg/ml. Again, as above, the complexity of the samples was rather low.

The same chemistry was used to coat slides with avidin and then bind randomly biotinylated antibodies (Rowe-Taitt *et al.*, 2000; Wiese *et al.*, 2001; Delehanty and Ligler, 2002) or Fab fragments biotinylated in the hinge region (Rowe *et al.*, 1999). Streptavidin/avidin interaction with biotin, the strongest non-covalent binding process known with a binding affinity constant of about 10^{15} l/mol, is often used for immunoassay construction (Schetters, 1999). Using a fluorescent sandwich assay, analytes could be detected at a concentration of a few ng/ml.

We analysed and optimised various modifications of glass surfaces (Kusnezow *et al.*, 2003). Epoxy-silanization as well as mercapto-silanization with maleimido-succinimidyl crosslinker (aminoreactive NHS-surface) or amino-silanisation with maleimido-succinimidyl crosslinker (thiolreactive maleimid-surface) proved to be simple and cheap derivatization strategies. In our hands, they produced three to four times better signal-to-background ratios compared with poly-L-lysine slides as well as high signal intensities in the range of a few pg/ml at optimal antibody concentration. In agreement with our results, other studies also reported that epoxy-silanized slides possess the highest sensitivity for this type of surface modification (Li and Reichert, 2002; Seong, 2002).

Some researchers have suggested that non-homogeneous spot morphology and smearing effects are inherent drawbacks of one-dimensional surfaces (Li and Reichert, 2002; Schaeferling *et al.*, 2002). From our experience, however, spot quality is purely a matter of proper surface preparation, usage of an appropriate spotting buffer, the actual protein concentration as well as the subsequent processing of the microarrays. Addition of detergent to the spotting solution, for example, results in higher spot homogeneity. The roughness of silanized surfaces (Piehler *et al.*, 2000; Benters *et al.*, 2001) that arises from inevitable oligomerization and formation of multiple silane layers has been hinted at as another source of inhomogeneity. However, it is limited to the nanometer scale and should therefore have no effect on the imaging process.

One problem caused by silanized surfaces is protein denaturation due to the surface hydrophobicity. For this reason, all the surface modifications discussed here require the addition of protective substances to the spotting buffer such as glycerol (MacBeath and Schreiber, 2000), disaccharides [trehalose (Kusnezow *et al.*, 2003), saccharose (Avseenko *et al.*, 2001)] or polyethylene glycol (PEG) of low molecular weight (Lee and Kim, 2002). Hydrophobic surfaces, however, exhibit a higher degree of unspecific

binding in comparison to hydrophilic support media (Piehler *et al.*, 1996, 2000). Therefore, these surfaces might be less suited to systems in which highly complex samples should be analysed, such as protein lysates from cell lines or tissues. Moreover, there could be a large steric influence on binding events due to the close proximity of the surface and the sensor molecules.

Two-dimensional coatings

This type of surface modification was used for years mainly for medical applications like the enhancement of bio-compatibility of implants and drug delivery systems (Bures *et al.*, 2001; Kumar *et al.*, 2001). PEG chemistry in particular offers a large variety of added functional groups and many PEG derivatives are commercially available. The main advantage of PEG-treated surfaces is their very low unspecific protein binding (Piehler *et al.*, 1996; Jo and Park, 2000). The actual reason for this is not fully understood. Also, the large spacer molecule, positioned between antibody and support matrix, helps avoiding steric interference and results in a higher analyte capture capacity (Weimer *et al.*, 2000). In our hands, PEG-modified surfaces yielded not only better absolute signal intensities than silanized surfaces but improved especially the binding of very large analytes (e.g. Fig. 3). Poly-L-lysine-grafted polyethylene glycol copolymers (PLL-g-PEG) exhibited extremely low unspecific protein binding (Kenausis *et al.*, 2000; Huang *et al.*, 2001b). Biotinylated proteins (Ruiz-Taylor *et al.*, 2001) as well as antibodies and Fab fragments (Peluso *et al.*, 2003) were attached to slides coated first with biotin and then with streptavidin.

Another class of matrix is the so-called self-assembled monolayer (SAM), formed usually by the spontaneous adsorption of alkanthiols on gold surfaces. However, other surfaces are also used (for review, see Schaeferling *et al.*, 2002). Alkanthiols that terminate in short PEG groups were shown to prevent effectively the unspecific adsorption of proteins (Zhang *et al.*, 2001a,b). Upon derivatization with reactive groups, proteins can be coupled in a desired manner.

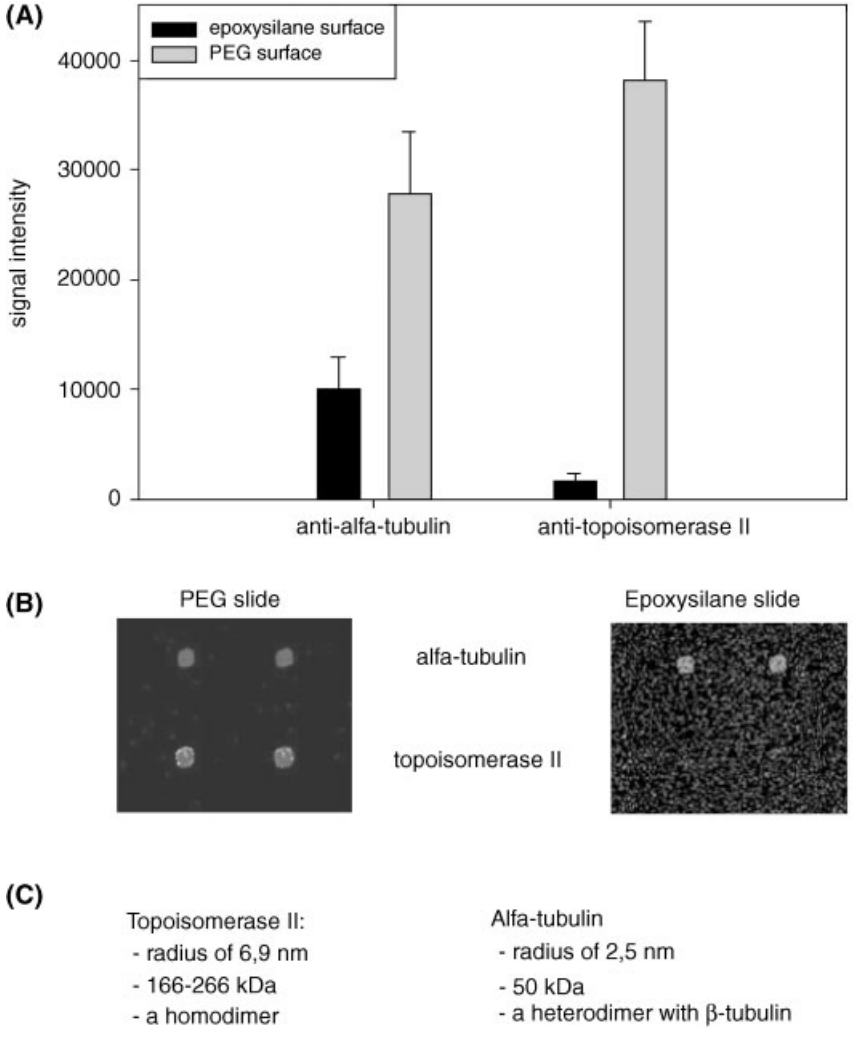

Figure 3. Comparison of epoxysilane surfaces and PEG-treated slides. Anti-α-tubulin and anti-topoisomerase II antibodies were printed on slides coated by the respective procedure. Incubation was with a Cy3-labelled protein lysate obtained from a colon carcinoma cell line (HT29). (A) Average signal intensity of both antibodies on either surface. On the epoxysilane slides, topoisomerase II produced a very weak signal only. Typical images are shown in (B). (C) A few biochemical parameters of the proteins.

Since too high a density of reactive groups may have negative effects, functionalized and non-functionalized polymers are usually mixed prior to surface derivatization, thereby controlling the density of immobilised antibodies (Knoll *et al.*, 1997; Li *et al.*, 2002). Since SAM allows the preparation of surfaces with well-defined topographic properties, it is used for the production of protein microarrays analysed with atomic force microscopy. Jones *et al.* (1998) developed an immunoassay based on the arraying of rabbit IgG molecules, using spot sizes of 7.5 μm. Upon addition of a secondary anti-rabbit antibody, binding could be detected by atomic force microscopy as an increase in the height of the spots. Lee *et al.* (2002) constructed protein arrays with 100–350 nm feature size for studies on antibody–antigen interactions and cell adhesion. The SAM approach can also be advantageous for fluorophor detection. Planar waveguide technology, for example, requires a homogeneous surface of defined thickness, since excitation of fluorescent labels occurs only within a short distance of the actual surface (Pawlak *et al.*, 2002).

Three-dimensional surfaces

A feature critical to all the microarray surfaces described above is their protein binding capacity. At best, spotting produces a monolayer of active proteins. For the same reason, three-dimensional, dendrimeric surfaces have been suggested for DNA-microarrays (Beier and Hoheisel, 1999; Benters *et al.*, 2001). Apart from the sensitivity, the dynamic range of measurement also increased. Immobilization strategies for protein arrays include coatings with various branched polymers as well as filter membrane surfaces (Stillman and Tonkinson, 2000).

One of the first demonstrations of the potential of protein microarrays used covalent attachment of proteins in tiny gel pockets, which in turn were attached to the glass surface. This approach was developed in the group of Andrei Mirzabekov (Guschin *et al.*, 1997; Vasiliskov *et al.*, 1999; Arenkov *et al.*, 2000) as an extension of similar work with DNA arrays and was applied to various types of immunoassays, enzymatic reactions as well assays with live cells (Barsky *et al.*, 2002). The three-dimensional matrix structure increased the loading capacity, reduced protein denaturation because of the homogeneous aqueous environment, and exhibited limited unspecific binding. However, the three-dimensional gel structure represents a barrier for diffusion and requires very long incubation times to achieve thermodynamic equilibrium, especially for low abundance proteins (Arenkov *et al.*, 2000). Another disadvantage is the complicated manufacturing process. Angenendt *et al.* (2002) simplified the procedure by pouring the polyacrylamide gel onto the entire slides instead of applying it to defined positions only. Ready-made polyacrylamide slides, called HydroGel, are available from Perkin-Elmer Life Sciences. Proteins are bound by adsorption to a hydrophilic matrix. Miller *et al.* (2003) compared the performance of HydroGel slides with poly-L-lysine slides, the latter additionally coated with a photo-reactive crosslinker (HSAB slides). When applied to protein profiling of prostate cancer and control sera, a six-fold higher signal-to-noise ratio was obtained on HydroGel compared with the HSAB slides.

As an alternative method, activation of an on-chip agarose gel with sodium metaperiodate was suggested (Afanassiev *et al.*, 2000). However, the achieved signal-to-noise ratios were rather disappointing. Nitrocellulose-coated FAST slides from Schleicher and Schuell (Stillman and Tonkinson, 2000; Beator, 2002) are another popular support matrix for protein arrays. The main reason for this is their enormous binding capacity (Kukar *et al.*, 2002), reflected in much higher signal intensities compared to one-dimensional surfaces (Joos *et al.*, 2000; Madoz-Gurpide *et al.*, 2001; Kukar *et al.*, 2002; Kusnezow *et al.*, 2003). Less than 1000 molecules of prostatic-specific antigen (PSA) could be detected in a single spot, taking advantage of a signal amplification system (Paweletz *et al.*, 2001). FAST slides seem to be a reasonable solid support for studies in which protein lysates of biological samples are spotted and subsequently probed with particular antibodies (Madoz-Gurpide *et al.*, 2001; Paweletz *et al.*, 2001). The surface is also attractive for detection by mass spectrometry due to the high binding capacity (Borrebaeck *et al.*, 2001; Tonkinson and Stillman, 2002). However, it is not suitable for analyses in which the microarrays are incubated with highly complex protein populations, since unspecific binding to the nitrocellulose is a significant problem under these conditions (Jones, 1999a,b). Knezevic *et al.* (2001) arrayed 368 antibodies on FAST slides in order to analyse protein expression in the microenvironment of squamous cell carcinoma of the oral cavity. Biotinylation of protein extracts with subsequent signal amplification was used for the analysis. Only 14% of the spotted antibodies produced a signal above background and a fifth of these signals gave no reasonable results due to the high degree of variation between individual experiments.

There is a limited number of other polymer based three-dimensional surfaces available (Schaeferling *et al.*, 2002). Benters *et al.* (2001), for instance, suggested a series of polyamino-functionalized dendritic macromolecule which were subsequently modified with homobifunctional crosslinkers. Prolinx Inc. and Accelr8 Technology Corporation offer slides coated with three-dimensional polymers, the former modified with salicylhydroxamic acid functional groups. Salicylhydroxamic acid forms a stable complex with phenyldiboronic acid, which is provided in the form of a protein-modifying reagent.

ATTACHMENT OF ALTERNATIVE SENSOR MOLECULES: RECOMBINANT ANTIBODIES, AFFIBODIES AND APTAMERS

Taking into consideration the fact that the wide concentration range of proteins will require for each antigen two or more receptors with different dissociation constants, the number of sensor molecules needed for an analysis of the entire proteome—estimated to consist of 100 000 to several million molecules—will be huge (Hayhurst and Georgiou, 2001). Classical antibody generation strategies based on animal immunization will not meet such a demand and it seems likely that future microarrays will be based on recombinant antibodies (Borrebaeck, 2000; Hayhurst and Georgiou, 2001; Siegel, 2002), engineered microbial

proteins such as affibodies (Nord *et al.*, 1997) or short single-stranded nucleic acids with protein binding properties, known as aptamers (Brody and Gold, 2000; Hesselberth *et al.*, 2000; James, 2001).

While directed attachment of a collection of native proteins is not a trivial issue, recombinant fusion antibodies and affibodies offer more and better opportunities to immobilize them in a directed manner. Zhu *et al.* (2001) produced fusion proteins with glutathione-*S*-transferase and a His_6 tag and spotted them on functionalised glass slides. *In vitro* protein expression of His-tag fusion proteins and preparation of protein *in situ* arrays (He and Taussig, 2001) may offer another option. In comparison to aldehyde slides, site-specific attachment on nickel-coated slides demonstrated superior signal intensity. However, the stability of the complex depends on the pH, a fact that can make applications difficult (Paborsky *et al.*, 1996). To overcome this, Lesaicherre *et al.* (2002) proposed a new strategy for a site-directed attachment of fusion protein using biotinylated affinity tags. Fusion proteins containing an intein-tag with chitin binding domain were purified on columns filled with chitin beads and biotinylated cysteine. Disruption of the protein–intein connection produced biotinylated proteins, which were spotted on avidin-coated slides. A similar principle was used by NextGen Sciences, expressing fusion proteins containing a tag of 15 amino acids. After specific biotinylation of this peptide by biotin ligase, the recombinant proteins could be directly spotted on streptavidin/avidin surfaces. Biotinylated fusion proteins represent a good alternative to covalent immobilization due to the strong interaction of biotin and streptavidin/avidin. Additionally, the biotinylation machinery of some microbial organisms could be used to produce biotinylation affinity tags *in vivo* (Nilsson *et al.*, 1997). Thereby, cell lysates could be printed directly onto microarray surfaces without laborious protein purification.

Another method to obtain a directed and stable attachment of recombinant proteins was reported by Hodneland *et al.* (2002). A recombinant calmodulin fused with serine esterase cutinase was immobilized to glycol-terminated SAM presenting a phosphonate ligand, which mimics a transition state product of the cutinase and binds covalently to the enzyme's active centre. Also, the generation of libraries of antibodies fused to a maltose binding protein (Bach *et al.*, 2001) was reported. An advantage of recombinant sensor molecules is the fact that they are smaller than antibodies. Consequently, a closer packing is possible, potentially resulting in higher immunoreactivity as well as reduced steric interference (Borrebaeck *et al.*, 2001; Peluso *et al.*, 2003).

Aptamers make a strong claim for their use as sensor molecules. Highly complex aptamer libraries (10^{13}–10^{17} different nucleic acid molecules) can track down targets by means of *in vitro* evolution (Brody and Gold, 2000). Using an aptamer-based assay, IgE molecules could be detected at an extraordinary sensitivity of 37 zmol (Hesselberth *et al.*, 2000). In an aptamer microarray scenario, aptamer–ligand complexes will be photo-crosslinked. A subsequent stringent wash will reduce non-specific binding and background. Aptamer microarrays could be constructed using classical DNA microarray surfaces or even be synthesized directly on the chip (James, 2001).

INCUBATION PARAMETERS

The history of miniaturized immunoassays started in the late 1980s, when Roger Ekins and coworkers created the first microspot multi-analyte immunoassay (Ekins *et al.*, 1990a; Ekins, 1998). Stemming from this was the ambient analyte theory (Jackson and Ekins, 1986; Ekins, 1989, 1994; Ekins *et al.*, 1990b), which describes microarray-based molecule interaction. Under the condition that the formation of antigen–antibody complexes does not significantly deplete the initial concentration of analyte, this theory predicts a much higher sensitivity of microspot multi-analyte immunoassays than conventional ELISAs and radio-immunoassays can deliver. It suggests a detection limit of approximately 10^{-17} M for a microarray of highly affine antibodies bound in a monolayer on spots of 100–1000 μm^2. Current microarray systems have spots with diameters of about 100–400 μm and thus fail to meet these ambient analyte conditions. Miniaturization of the spots will therefore not only positively impact the multiplex factor of assays (Laurell and Marko-Varga, 2002), but also improve sensitivity. New developments in bio-molecule printing technologies open enormous possibilities in this direction. Okamoto *et al.* (2000) applied ink-jet technology for microarray printing, for example. As opposed to conventional contact printing, this technology can produce spots with a diameter of 25 μm (Harris *et al.*, 2000). Electrospray deposition—used originally for molecule ionization—can also be applied to microarray construction, with feature sizes down to few micrometers (Morozov and Morozova, 1999; Avseenko *et al.*, 2001, 2002). Currently, the spots produced by this approach have an irregular shape, however. Protein microarrays used in experiments based on detection by atomic force microscopy were produced by lithographic printing. They have a resolution in the nanometer range (Bernard *et al.*, 2000) and approach the range of depositing individual molecules (Michel *et al.*, 2001).

Most fluorescent dyes currently used are hydrophobic substances, which substantially decrease the solubility of proteins and interfere at high incorporation levels with antigen antibody binding (Patton, 2000), thereby influencing adversely signal intensity. Because of this, some researchers have argued against fluorescent labelling (MacBeath, 2002). However, as known from DNA microarrays, overall performance depends not necessarily on individual parameters or compounds but requires an integrated system, in which all components such as chip surface, labelling procedure and detection system are matched. Still, other detection modes—even the 'old-fashioned' radioactive labelling—might be superior to fluorescence or better suited for particular applications.

According to theory, the higher the fractional occupancy of antibodies the lower will be the unspecific binding of proteins to the spots, in consequence yielding higher sensitivity. On the other hand, one needs to consider that the increase in complexity of multi-analyte experiments is bound to increase background in and between the spots. With respect to incubation time, longer periods are required for achieving equilibrium in microarray assays (Butler, 2000). A strong dependence of classical immunoassays in microtitre plates on diffusion is well known and can be

overcome by intensive shaking, which strongly reduces the required incubation time. Microchip formats suffer even more from diffusion constraints. Modelling of DNA microarray experiments demonstrated that low-abundance analytes would require many days to reach equilibrium at standard hybridization conditions (Bhanot *et al.*, 2003). For protein interaction, the result is likely to be similar, an assumption supported by kinetic experiments that we performed with antibody microarrays. In a solution containing only few picograms per millilitre of antigen, 4–5 days were required in order to achieve maximal sensitivity (unpublished data). Since binding affinity constants of antigen–antibody interactions are relatively low (usually 10^8–10^{10} mol/l) and may decrease upon antibody immobilization, there is a strong demand for technologies enabling a reliable non-laminar mixing of very small volumes. Advalytix (Munich, Germany), for example, developed an agitation system for mixing 20 and 150 µl of fluid on a microarray (Scriba *et al.*, 2002). Nanopumps integrated in a cover slip substitute produce surface acoustic waves which cause mixing of the solution. Applied in protein microarray analyses, this system resulted in shorter incubation times and much higher signal intensities. BioMicro Systems fabricated a system for DNA microarrays based on two air-driven bladders that continuously mix 35–40 µl of hybridization solution (Adey *et al.*, 2002). Gains in sensitivity across a DNA-microarray of 6912 spots were 2- to 3-fold.

CONCLUSION

Microarray-based immunoassays are currently in a state of early development and further progress and refinements are needed before large data sets will be produced by such means. However, many of the basic obstacles to such ends have been identified and are being worked on. Therefore, we are confident that many technical problems will be circumvented in due course. Then the bottleneck will move from mere production issues toward the ability and capacity to identify and isolate suitable binder molecules. In this respect, microspot immunoassays follow a path that is similar to the development of DNA microarray analysis. Given the existing experience in microarrays on the one hand and keeping in mind the higher degree of complexity of the protein world and the lack of an *in vitro* amplification method equivalent to PCR, a period of several years may be required before chip-based proteomics will work as well as DNA microarrays do today.

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