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Antibody microarrays: An evaluation of production parameters

Antibody microarrays could have an enormous impact on the functional analysis of cellular activity and regulation, especially at the level of protein expression and protein-protein interaction, and might become an invaluable tool in disease diagnostics. The array surface is bound to have a tremendous influence on the findings from such studies. Apart from the basic issue of how to attach antibodies optimally without affecting their function, it is also important that the cognate antigens, applied within a complex protein mixture, all bind to the arrayed antibodies irrespective of their enormous variety in structure. In this study, various factors in the production of antibody microarrays on glass support were analysed: the modification of the glass surface; kind and length of cross-linkers; composition and pH of the spotting buffer; blocking reagents; antibody concentration and storage procedures, in order to evaluate their effect on array performance. Altogether, data from more than 700 individual array experiments were taken into account. In addition to home-made slides, commercially available systems were also included in the analysis.

1 Introduction

DNA microarrays have become an essential tool in the functional interpretation of sequence information yielded from the various genome projects. Many aspects of modulation and regulation of cellular activity at the level of nucleic acids can be investigated with this technology. A major area of analysis are studies of the variations in gene expression by comparing transcript levels present in cells from different tissues or growth conditions. However, the data provide only a limited insight into the process of actual protein expression and even less information on protein-protein interaction or the proteins' biochemical activity. Consequently, there is a strong demand for analysis procedures at the protein level that correspond in performance to the kind of studies possible on DNA microarrays [1–3]. As a matter of fact, even higher capabilities will be required from such techniques. The human proteome is much more complex in composition than the coding portion of the genome. Estimates range

from 100 000 to more than 1 500 000 different protein molecules present in a cell [3, 4] resulting from domain shuffling effects and post-translational modifications.

The current standard for the elucidation of global variations in the expression of proteins is still 2-DE [5–7]. However, this process is time-consuming and expensive. This is a critical obstacle to routine application in areas such as diagnostic assays, for example, in which relatively few molecules of high relevance to the constitution of a disease are analysed. In addition, reproducibility is problematic, even though precast gels and commonly used reagents, protocols and hardware components have led to improved performance [8]. Although still in a developmental stage, antibody microarrays might be a viable alternative, once a number of procedural and technical aspects have been advanced and improved to make the arrays a reliable tool [9]. The broad range of antibody specificity and affinity complicates detection of proteins by antibody-antigen interactions. In addition, these binding affinities are generally weaker than DNA-DNA or RNA-DNA interactions [10]. Therefore, the selection of suitable sensor molecules is an important issue. Also, the microarray surface is critical in this respect. While it is difficult to define general immobilisation strategies that do not discriminate between proteins, the structural similarity of antibodies mollifies this aspect. Nevertheless, the solid support has profound consequences on the quality of microarray analysis, since it influences not only the efficiency of antibody attachment but also the degree of nonspecific binding, for example, and the accessibility of the

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Abbreviations: APTES, (3-aminopropyl)trimethoxy silane; **GFP**, green fluorescent protein; **GPTS**, (3-glycidoxypropyl)trimethoxy silane; **KLH**, keyhole limpet hemocyanin; **MPTS**, (3-mercaptopropyl)trimethoxy silane; **NHS**, *N*-hydroxysuccinimide; **PL**, poly-L-lysine

antibodies to the antigens, which differ widely in structure. The use of different surfaces has been reported. On poly-L-lysine slides, for example, several antibody-antigen interactions could be detected in concentration as low as 10 ng/mL [11] and also other surfaces like aldehyde or nitrocellulose coated slides were used with some success [*e.g.*, 12, 13]. However, generally only a small number of rather similar antigens were looked at and no in-depth comparison of performance parameters was carried out.

Here, we present data based on the analysis of overall more than 700 slides. Aiming at the application of antibody microarrays for profiling protein expression and protein clustering, we compared different strategies of fabricating such microarrays on glass-slides using standard microarray instrumentation and fluorescent labelling of the antigens. We concentrated our efforts on the glass-slide format, since it is compatible with hardware and software tools existing for DNA microarrays, thus facilitating preparation, processing and data evaluation. Several derivatisation strategies of the glass-slides and buffering systems were tested, optimised and compared to commercially available systems. Also, the effect of different blocking reagents and the influence of slide storage were examined. Analysis was performed with protein antigens of different sizes and at different concentrations. The information permits an evaluation of the consequences of production conditions on the results, thus improving quality and reliability of assays on antibody microarrays.

2 Materials and methods

2.1 Materials

All chemicals and solvents were purchased from Fluka (Taufkirchen, Germany), Sigma-Aldrich (Munich, Germany) or SDS (Peypin, France), unless stated otherwise, and used without additional purification. Untreated slides were purchased from Menzel-Gläser (Braunschweig, Germany); amino-silanised slides from Sigma and Corning (Schiphol-Rijk, The Netherlands); FAST™ slides from Schleicher & Schuell (Einbeck, Germany); QMT epoxy slides from Quantifoil Micro Tools (Jena, Germany); aldehyde slides and ArrayIt spotting solution from TeleChem (TeleChem International Sunnyvale, CA, USA). (3-glycidoxy-propyl)trimethoxy silane (GPTS), (3-aminopropyl)trimethoxy silane (APTES), (3-mercaptopropyl)trimethoxy silane (MPTS), BSA, milk powder and TopBlock solution were obtained from Sigma-Aldrich. 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylhydrazide dioxane and succinimidyl-4-[*N*-maleimidomethyl]-cyclohexane-1-carboxy-[6-amidocaproate] were purchased from Pierce (Rockford, IL, USA); 3-maleimidopropionic acid *N*-hydroxysuccinimide

ester, 6-maleimidohexanoic acid *N*-hydroxysuccinimide ester, 11-maleimidoundecanoic acid *N*-hydroxysuccinimide ester were obtained from Sigma.

Immunoglobulins and corresponding antigens were obtained from the following companies: monoclonal anti green fluorescent protein (GFP) antibody (IgG1k isotype) from Hoffmann-La Roche (Mannheim, Germany); monoclonal antihuman interferon-γ antibody (I5521; IgG2a isotype) and recombinant human interferon-γ (I3265) from Sigma-Aldrich. Keyhole limpet hemocyanin (KLH), polyclonal anti-KLH antibody, thyroglobulin and polyclonal antithyroglobulin antibody were a kind donation of Eurogentec (Seraing, Belgium); monoclonal anti-p16 antibodies (IgG1 isotype) and recombinant p16 were a gift from MTM Laboratories (Heidelberg, Germany).

2.2 Surface derivatisation of glass slides

Untreated slides were washed with ethanol and then etched by immersion in 10% NaOH at room temperature for 1 h. Subsequently, the slides were placed again in 10% NaOH and cleaned by sonification for 15 min. They were rinsed four times in water, washed twice in ethanol and derivatised in the appropriate solution at room temperature for 1 h, again followed by a sonification step. The following derivatisation solutions were used: GPTS slides: 2.5% GPTS, 10 mM acetic acid in ethanol; APTES slides; 5% APTES in 95% ethanol/water; MPTS slides: 1% MPTS, 10 mM acetic acid in ethanol; poly-L-lysine slides: 0.01% poly-L-lysine solution, $0.1 \times$ PBS buffer ($1 \times$ PBS: 137 mM NaCl, 2.7 mM KCl, 10 mMM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). After silanisation, GPTS-treated slides were washed thoroughly with ethanol, while MPTS slides were additionally rinsed with 16 mM acetic acid in ethanol. APTES and poly-L-lysine slides were washed first with water and then twice with ethanol. All slides were dried with nitrogen. The APTES slides were finally baked at 110°C for 15 min, poly-L-lysine at 45°C for 30 min.

2.3 Addition of cross-linkers

Aminosilane (APTES), mercaptosilane (MPTS) and poly-L-lysine slides were additionally derivatised with different cross-linkers. All cross-linkers were diluted in DMF and stored at a concentration of 200 mM at 4°C. Prior to use, the cross-linkers were diluted in DMF to a final concentration of 20 mM. Fifty µL of the respective cross-linker solution were pipetted onto the slide surface and covered with a glass coverslip that had been cleaned with ethanol. The slides were incubated at room temperature for 3 h. Subsequently, excess of cross-linker was removed by washing twice with DMF and twice with ethanol, before the slides were dried by centrifugation.

2.4 Preparation of antibodies

Various ingredients and different salt concentrations contained in the buffer of commercially obtained antibodies may have an influence on the efficiency of spotting. To avoid this, all antibodies were transferred into $0.25 \times$ PBS and 0.02% sodium azide by means of the Microcon centrifugal filter device system (Millipore, Bedford, USA). The final concentration of the antibodies was determined with the NanoOrange protein quantification kit (Molecular Probes, Eugene, OR, USA) and adjusted to 8 mg/mL. Oxidation and reduction of antibodies were carried out according to standard protocols [14]. Briefly, for oxidation 4–5 mg/mL of antibody in $1 \times$ PBS and 10 mM sodium periodate were incubated 30 min in the dark. For reduction, 2–4 mg/mL of antibody in $1 \times$ PBS, 10 mM EDTA and 50 mM cysteamine were incubated at 37°C for 90 min. The antibodies were purified with Micro Bio-Spin30 columns (Bio-Rad, Munich, Germany) and concentrated with the Microcon filter device system.

2.5 Fabrication of antibody arrays

The following buffers were used in the experiments on spotting solutions: carbonate buffer (100 mM NaCO$_3$, 300 mM NaCl, pH 8.5), PBS buffer ($1 \times$ PBS, 300 mM NaCl, pH 7.4), acetate buffer (100 mM NaAc, 300 mM NaCl, pH 5.5), and citrate buffer (100 mM sodium citrate, 300 mM NaCl, pH 4.5). PBS buffer supplemented with 0.5% trehalose was used as spotting buffer in all other experiments. Only fresh silanised and one month old poly-L-lysine slides were used, unless stated otherwise. Spotting of antibodies was done with an SDDC-2 Micro-Arrayer (Engineering Services, Toronto, Canada) or a MicroGrid-II system (BioRobotics, Cambridge, UK) and SMP3 pins (TeleChem). The slides were incubated at 4°C overnight and subsequently blocked by a 4–5 h incubation at room temperature in $1 \times$ PBS supplemented with BSA, TopBlock or milk powder, respectively, at the concentrations indicated in Section 3. For long-term storage of spotted slides, some of the slides were first blocked in 3% TopBlock, 1% trehalose and 0.02% sodium azide in $1 \times$ PBS, centrifuged dry and stored at 4°C. Others were blocked without the addition of trehalose and stored in a humid chamber at 4°C.

2.6 Antigen labelling

Antigen mixtures of 1 mg/mL were labelled with the monofunctional *N*-hydroxysuccinimide NHS-ester of the dyes Cy5 or Cy3 (Amersham Biosciences, Freiburg, Germany), respectively, as recommended by the manufacturer. Unreacted dye was blocked from further reaction by adding hydroxylamine to a final concentration of 1 M. Labelled antigen solution was dialysed three times for 12 h each against PBST buffer ($1 \times$ PBS, 0.05% Tween 20) in Slide-A-Lyser Dialysis Cassettes or Slide-A-Lyser Mini Dialysis Units (Pierce Biotechnology). For long-term experiments, antigens were labelled once only, aliquoted and stored at -20°C to avoid any influence of labelling variation.

2.7 Incubation

Incubation of the microarrays with antigens occurred at 4°C overnight either in slide containers (if several slides were incubated simultaneously) or individually in EasiSeal chambers (Thermo Hybaid, Ulm, Germany) covered with Hibri-Slip (Sigma-Aldrich). The concentration of antigens was usually 100 ng/mL. For the experiments with cross-linkers, γ-interferon, thyroglobulin and KLH were mixed at a ratio of 1:2:3 and labelled with Cy3 so that the antigen concentrations were 50, 100 and 150 ng/mL, respectively. This ratio was found to be optimal to produce similar signal intensities on the antibodies. After incubation, the slides were washed three times with $2 \times$ PBS for about 3 min and centrifuged at 500 rpm for 5 min to dry.

2.8 Scanning and evaluation

Fluorescence signals were detected on a ScanArray5000 unit (Packard, Billerica, MA, USA) and analysed with the GenePix software package (Axon Instruments, Union City, CA, USA). The slides were first scanned at conditions optimal for each individual slide and additionally analysed at a setting identical to all slides of the respective experimental series. Background signal was always determined at several positions across a microarray surface. The results were stored and managed in an appropriately structured database. The complete data set analysed consists of the signal intensities of some 36 000 spots plus their local background as well as 21 000 separately measured background values. Each data point presented in this report represents the average of 6–12 individual measurements obtained from at least three slides.

3 Results

3.1 Assessment of strategies for the attachment of antibodies to a glass surface

Initially, we attached the antibodies to the solid support in a directed orientation *via* their carbohydrate groups after activation with sodium metaperiodate, spotting them on

hydrazide, aminosilane and poly-L-lysine surfaces as reported [15–17]. As an alternative procedure, cysteines of the antibodies were activated by a reduction with cysteamine, subsequently spotting them on maleimide surfaces. In the former process, the binding of the antibodies occurs *via* the aldehyde groups of the carbohydrates, while in the latter case the thiol groups react with the surface. However, in our hands these immobilisation techniques produced slides that were not very different with respect to signal-to-background ratios obtained when nonactivated antibodies were spotted directly (see below). As a matter of fact, the activation step created difficulties. First, in our hands the treatment and subsequent purification steps led to a loss of up to 40% of an antibody. Second, the antibodies had to be activated directly prior to spotting but could not be kept in this state for a long time, again wasting valuable resources. Since similar problems could be expected with other attachment strategies that require a pretreatment of the antibodies, we looked further only into immobilisation strategies that lack prior antibody modification.

In a screen of possible surface modifications, we tested a variety of potentially appropriate surfaces. In this phase, only antibodies against small antigens such as green fluorescent protein (GFP), P16 and γ-interferon were used to exclude potential discrepancies in the data caused by the size of the antigens. The binding strategies could be roughly subdivided into three classes: (1) attachment by adsorption, (2) coupling *via* amino groups and (3) binding by the thiol groups of the antibodies (Table 1). Quality was assessed by the criteria of signal intensity, background level and spot quality. For comparison of signal intensities, dilution series of 5 mg/mL down to 10 μg/mL of the antibodies were spotted. For each experimental condition, at least three replications were done to assure reproducibility. The signal-to-background ratio obtained at the antibody concentration that was optimal for the respective system was scored and formed the basis of the comparison. In addition to the ratio, the absolute signal intensities – after substraction of the background signals – were scored, since they also define the dynamic range possible. Finally, spot morphology was evaluated visually. In an amalgamation of these three types of data, the slides were binned into five quality categories (Table 1). Although only of a semiquantitative nature, the authors can provide upon request the actual values or spot images resulting from these experiments.

3.1.1 Binding by absorption

In the adsorption group, home-made poly-L-lysine (PL) and APTES slides were tested as well as slides coated with aminosilane commercially available from Sigma and

Corning. PL slides were used because of their history as a protein microarray surface and the fact that protein profiling measurements and characterisation of autoantibody responses have been reported using this support [18, 19]. Overall, signal intensity, background and spot morphology on PL slides were found also in our initial analysis to be of sufficient quality. Therefore, this support was defined as the standard surface, to which all other surfaces were compared, and included in all our experiments. Aminosilane derivatised glass surfaces were found to be less suited for protein microarray experiments for their high background signal, which was about 4–5 times more intense than the background on PL slides, with concurrently a lower signal intensity.

3.1.2 Binding by amino groups

For the analysis of antibody binding *via* amino groups of their lysines or arginines, MPTS surfaces were derivatised with a heterobifunctional cross-linker which contains a sulfhydryl reactive maleimide group at one end and an amine reactive NHS ester at the other end (Table 1). Initially, a relatively short maleimide-NHS cross-linker was used (C1; see Table 1). At a later stage, optimisation of the cross-linker length was performed (C2–C4; see Section 3.4). MPTS surface derivatised with cross-linkers are frequently used for the production of biosensors [16, 20]. In our hands, it was found important to incubate mercaptosilane slides in acidic buffer in the terminal phase of their preparation.

Binding antibodies *via* their amino groups was also achieved on home-made or commercial GPTS slides. Application of the epoxysilane-coated surface had been reported in the context of a microwell kinase chip and exhibited a very high binding capacity [21]. The performance of GPTS slides could be improved by increasing the concentration of epoxysilane in the derivatisation solution to 2.5%, instead of the usual 1%, thereby obtaining 40–50% higher signal intensities. Even higher concentrations such as 5% epoxysilane, however, produced similar signal intensities but significantly higher background. Aldehyde slides from TeleChem compared unfavourably to the other systems in terms of both signal and background intensity.

3.1.3 Binding by thiol groups

In contrast to the MPTS-C1 slides, the maleimide-NHS cross-linker binds in the reverse orientation on the APTES and PL surfaces. Therefore, APTES-C1 and PL-C1 represent maleimide surfaces. The cross-linker interacts with the amino group on the glass slide while the antibodies

Table 1. Summary of an initial experimental comparison of various strategies of antibody attachment to glass slides. Signal intensities were compared only for small antigens such as GFP, P16 and γ-interferon to avoid size effects. For each surface, the best performing antibody concentration was taken into account. Three independent experiments were done for each condition. The slides were binned into five quality categories according to an assessment of signal-to-noise ratio, absolute signal intensity and spot morphology.

Surface derivatisation	Cross-linker	Abbreviation	Formula	Signal	Background	Spot quality
			Antibody binding by adsorption			
Poly-L-lysine[a]	None	PL		Standard +/−	Standard +/−	Standard +/−
Aminopropyl-trimethoxysilane	None	APTES		−	−	−
Aminosilane slides from *Sigma* and *Corning*			−/−	−	−	−
FAST slides from *Scheicher & Schuell*			−/−	++	−−	−
			Antibody binding *via* aminogroup of lysines or arginines			
Mercatopropyl-trimethoxysilane	Different maleimido-R-*N*-succin-imidylesters	MPTS-C1 MPTS-C2 MPTS-C3 MPTS-C4		+	+/−	+
Glycidoxypropyl-trimethoxysilane	None	GPTS		+	+/−	+
QMT Epoxy slides			−/−	+	+/−	+
TeleChem Aldehyde slides			−/−	+/−	−	+
Aminopropyl-trimethoxysilane	Different maleimido-R-*N*-succin-imidylesters	APTS-C1 APTS-C2 APTS-C3 APTS-C4		+	+/−	+/−
Poly-L-lysine	Different maleimido-R-*N*- succin-imidylesters	PL-C1 PL-C2 PL-C3 PL-C4		+	+/−	+/−
Mercatopropyl-trimethoxysilane	None	MPTS MPTS-C2 MPTS-C3 MPTS-C4		−	+ [b]	−/−
Aminosilane slides from *Sigma* and *Corning*	3-maleimido-propionacid-*N*-succin-imidylester	−/−	−/−	+	−	+/−

a) The antibody microarray quality obtained on PL slides was defined as standard (+/−), to which all tested immobilisation strategies were compared. A (−) or (−−) indicate worse results, while (+) and (++) stand for better performance. The raw data are available from the authors upon request.
b) MPTS slides were additionally blocked with methylmaleimide. For the surfaces typed in blue, more, quantitative data is presented below.

are attached by thiol groups of their cysteines. APTES-C1 was used by MacBeath *et al.* [22] to print small molecules that contained sulfhydryl groups. We performed a direct comparison of signal intensities obtained on untreated polyclonal anti-KLH and antithyroglobulin antibodies and material that had been activated by a reduction with cysteamine prior to spotting. Identical signal intensities could be obtained applying nonreduced antibodies at an only slightly higher concentration in the spotting solution.

A derivatisation with C1 cross-linker of the commercial aminosilane slides did not improve their performance in terms of signal-to-background ratio in comparison to home-made APTES-C1 slides, again because of considerably higher background. PL slides are believed to have a higher density of amino groups than APTES slides, from which one would expect a higher binding capacity. In spite of this, the cross-linker derivatised PL slides exhibited in all cases a slightly lower signal intensity compared to the corresponding APTES surfaces. Other parameters such as background and spot quality were similar for both surfaces.

In addition to the above procedure, attachment of antibodies *via* their thiol-groups was also performed by spotting them on unmodified mercaptosilane slides. These slides showed excellent, low background when blocked with methylmaleimide (see Section 3.2) but low signal intensity and very bad spot quality.

3.1.4 Initial conclusions

In summary, best results were obtained for APTES-C1, MPTS-C1, GPTS and PL surfaces. The first three surfaces displayed signal intensities comparable or higher than those on PL slides, with comparable background values and similar or even better spot morphology. Because of their superior performance in the initial assays, they were selected for a more detailed, quantitative evaluation.

3.2 Blocking

Unspecific background signal (antigen binding in the absence of antibody) is one of the most serious, if not the most severe, problem encountered in protein microarray technology. Already in this respect, it is different to cDNA-microarrays, which are less plagued by this problem. Initially, we tried to decrease the unspecific background signal by chemical modification of the surface coatings, incubating slides 30 min in the respective solution prior to a blocking reaction with 1% BSA. Practically

all these attempts failed, however. APTES-C1 and PL-C1 slides were treated with 10 mM mercaptoethanol or 10 mM cysteamine to block the maleimide group activity [22]. This procedure, however, significantly reduced the specific signal intensities while the background values were unchanged or even higher. Blocking of GPTS slides with 100 mM hydroxylamine actually increased background and the spot quality was affected very badly indeed. Blocking of NHS ester activities on MPTS-C1 slides with hydroxylamine or 1 M Tris did not produce significant differences of background or signal intensities. Only the background signal on unmodified MPTS slides was positively affected by an incubation in 5 mM methylmaleimide.

For improving blocking efficiency, we used BSA ($\sim$60 kDa) in concentrations of 1% and 3% in 1 $\times$ PBS and also looked at TopBlock, a mixture of small proteins ($\sim$3 kDa), at the same concentrations. TopBlock is more soluble than BSA. Typical results for APTES-C1 are shown in Fig. 1. On all surfaces analysed, background blocking was superior at the higher concentration of both BSA and TopBlock. While TopBlock did not affect the specific signal intensities obtained in the subsequent analyses, however, BSA reduced these signals, especially in case of large antigens such as thyroglobulin (670 kDa) or KLH (6000 kDa). In addition, the background produced on surfaces blocked with 3% TopBlock was about four times lower than results obtained with 3% BSA. The strong inhibitory effect on signal intensities was also observed with 4% milk powder, a classical blocking reagent. Therefore, 3% TopBlock solution was used as blocking reagent in all experiments reported here.
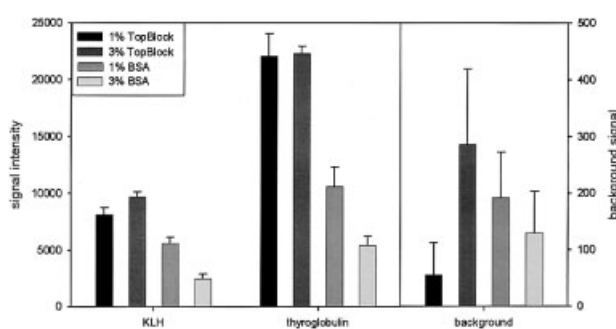

Figure 1. Blocking with 1% and 3% of TopBlock and BSA, respectively (left to right as indicated in the inlet). Typical results obtained on APTES-C1 surfaces are shown. KLH and thyroglobulin were used as antigens. In the left panel, the absolute signal intensities are shown. In the right panel, the absolute background signals are presented. Since background was very similar for both antigens, an overall average was calculated. Signal intensity is given in arbitrary units, which represent the actual output of the scanning device. Note the difference in the scaling of the left and right panel.

3.3 Spotting buffers

Spotting buffer composition can influence the protein-binding capacity of a surface, the stability of proteins and the quality of the spots produced. We tested on the different surfaces several spotting buffers (carbonate buffer, pH 8.5; PBS buffer, pH 7.4; acetate buffer, pH 5.5; and citrate buffer, pH 4.5). In addition, the ArrayIt buffer from TeleChem was included in the analysis as it is frequently used in cDNA microarray production. The effect of the supplements trehalose and glycerol was also investigated, compounds that prevent dehydration of the spotting solution and may improve the stability of antibodies.

Surprisingly, the pH seemed to have little effect. The best absolute signal intensities and signal-to-background ratios were obtained with PBS buffer supplemented with 0.5% trehalose, the ArrayIt buffer and citrate buffer with 0.5% trehalose (Fig. 2). From these, PBS buffer plus 0.5% trehalose was universally the best in terms of absolute signal intensity as well as signal-to-background ratio irrespective of the kind of surface. The concentration of the additive had a strong influence on the results. PBS buffer with 0.1% trehalose, for example, did not behave significantly differently to mere PBS buffer. A concentra-

tion of 2.5%, on the other hand, produced high background and smeared spots. Addition of 5% glycerol [12, 23] resulted in a signal of about half the intensity and produced spots of bad quality, an effect that actually got worse at higher concentrations (10% and 40%).

3.4 Optimisation of cross-linker length and dependency of signals on antigen size

Steric hindrance could influence the efficiency of antigen-antibody interaction thus causing a reduction of signal intensities. To examine if absolute signal intensities depend on antigen size and if the length of a cross-linker has a positive influence, APTES and MPTS surfaces were derivatised with four maleinimido-NHS cross-linkers of different length (C1 to C4; see Table 1 for structures). To minimise variations in reactivity of a functional group, cross-linkers C1 to C3 were selected, since they have a structurally similar link between the functional groups. APTES-Cn, MPTS-Cn as well as PL-C4 and unmodified PL and GPTS slides were coated with three polyclonal antibodies against antigens of very different molecular weight: γ-interferon (17 kDa), thyroglobulin (670 kDa) and KLH (6000 kDa). APTES-C4 slides exhibited the highest signal intensities for all antibodies. Consequently, the signal intensities obtained on the various surfaces were normalised against the corresponding signal intensities on the APTES-C4 slides (Fig. 3).

In the case of APTES-Cn slides, we could observe a clear increase of signal intensity with increasing size of the cross-linker. This effect was most pronounced for antibodies that bind large antigens such as KLH or thyroglobulin. Signal intensity more than doubled from the shortest to the longest cross-linker. Surprisingly, signals on the MPTS-Cn slides did not show such differences. The signal intensity for KLH, the largest antigen, was about half that obtained on the APTES-C3/C4 surfaces and did not improve with increasing length of the cross-linkers. Additionally, there was actually less background on slides containing the longest cross-linker (C4) possibly due to its structural difference. Also on other support media such as PL and GPTS slides, a strong dependency of signal intensity on antigen size was observed. On GPTS and PL slides, KLH produced about 30% of the signal obtained on APTES-C3 or APTES-C4 surfaces, while the signal for anti-γ-interferon was more than 60% as intense.
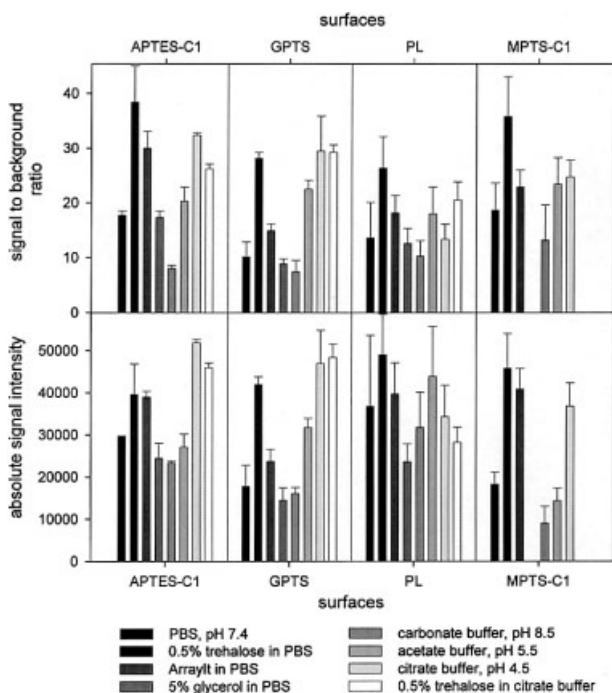
3.5 Optimal antibody concentration

To determine the optimal antibody concentration in the spotting solution, APTES-C4, MPTS-C4, GPTS and PL slides were analysed (Fig. 4). The relative signal intensities


Figure 2. Effect of spotting buffer. The values for the signal-to-noise ratios (upper row) and the absolute signal intensities (lower row) are indicated. Data are represented for anti-γ-interferon with an antibody concentration in the spotting solution of 1 mg/mL. Data for the citrate buffer with 0.5% trehalose and 5% glycerol in PBS buffer are absent for the MPTS-C1 surface.
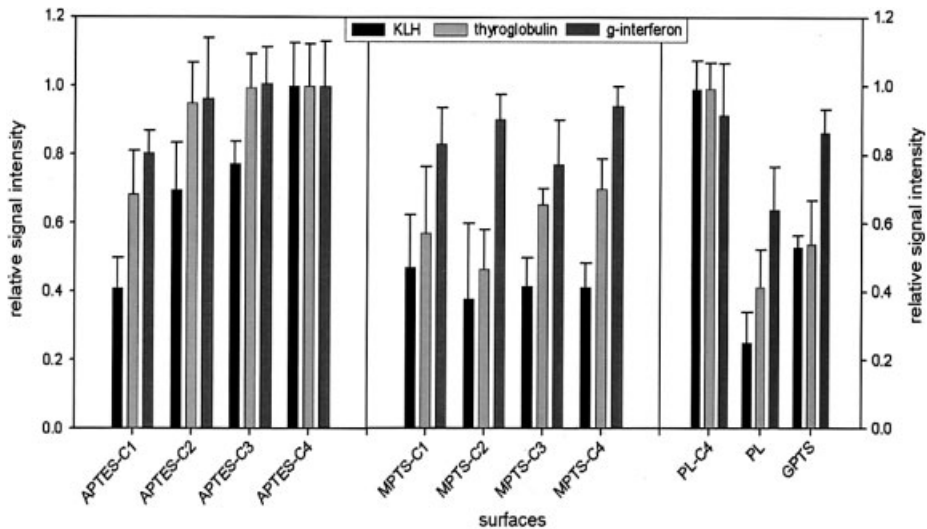
Figure 3. Dependency of signal intensity on cross-linker length and antigen size. APTES and MPTS slides were derivatised with cross-linkers of different length as reported in Section 3.4. Signal intensities were recorded with antigens that differ strongly in size (γ-interferon: 17 kDa; thyroglobulin: 670 kDa; KLH: 6000 kDa). All absolute signal intensities were normalised against the corresponding signal intensities on the APTES-C4 surface.
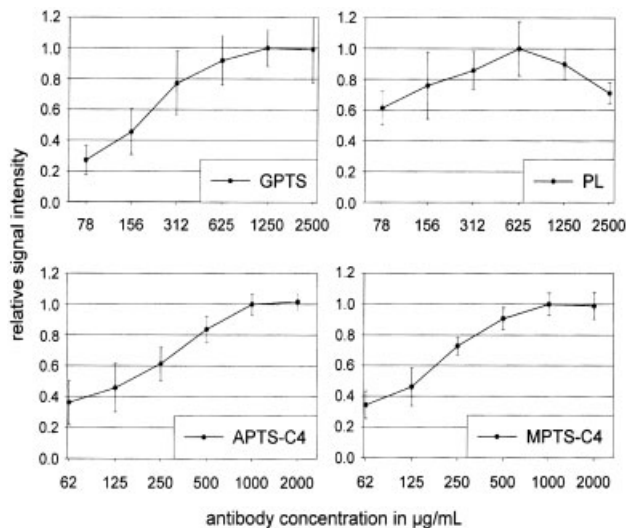

Figure 4. Effect of the antibody concentration in the spotting buffer on signal intensity. The highest signal intensity in each system was defined as 100%, relative to which changes in intensities are shown.

of spotted anti-γ-interferon, antithyroglobulin and anti-KLH antibodies were taken into account. Not surprisingly, saturation and even a subsequent reduction in signal could be observed. On PL slides, there was practically no signal left at an antibody concentration of about 5 mg/mL. The other three systems did not differ in performance between each other, reaching a broad optimum at an antibody concentration in the spotting solution of about 1 mg/mL. Much higher concentrations (3–5 mg/mL) had again an adverse effect on microarray performance.

3.6 Storage of coated glass slides

3.6.1 Storage of blank slides

The ability to store slides prior to spotting is an important time- and material-saving element in microarray technology, since it allows bulk production of slides and their subsequent continuous consumption in experimentation. It is known for DNA arrays that slides coated with poly-L-lysine, for example, actually mature with time, producing better results if left untouched for about one month before being used for array preparation. Here, freshly prepared APTES and GPTS slides were kept at 4°C under argon or in a normal atmosphere, respectively. They were used for antibody spotting within a day after preparation or after 2 weeks, 1 month, 2 months and 4 months (Fig. 5). Binding of cross-linker on APTES slides was carried out immediately prior to spotting. As a control, 1-month old poly-L-lysine slides from various preparations were used in parallel. All slides were scanned at identical scanner adjustments, using controls to assure that there was no difference in the sensitivity of the scanner during time.

Cross-linker-modified APTES slides only slightly improved their performance during the two month period of storage. No such effect could been seen when the slides were stored in an argon atmosphere. GPTS slides showed a strong increase in signal intensities after two months of storage under argon. Without argon, less increase in the slides' performance was observed. Analysis of particular antibody responses demonstrated that antibodies specific to a large antigen such as KLH were more affected.
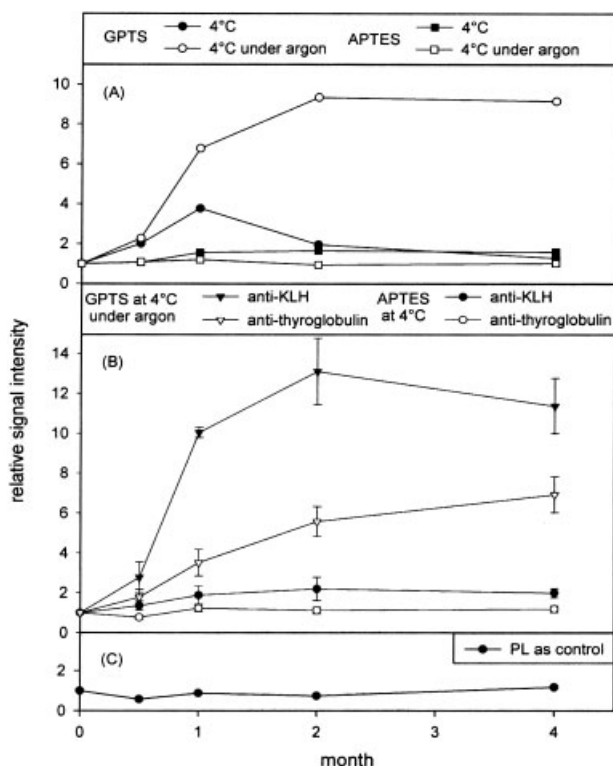
Figure 5. Long-term storage of slides prior to spotting. GPTS slides were treated with 1% epoxysilane at time point zero. Cross-linker derivatisation of the APTES slides was done directly prior to the spotting of antibodies. In (A), the effect of the atmosphere is shown. In (B), the influence of the antigen size and surface modification is demonstrated. Panel (C) represents control data obtained with PL slides during the period of the experiment in order to assure that variations are not caused by experimental biases such as denaturation of the proteins, bleaching or variation in the detection sensitivity, for example.

3.6.2 Storage of antibody-containing slides

Parallel to the above experiment, APTES-C1 and GPTS slides were used for spotting antibodies the day after the coating had taken place and stored at 4°C in dry conditions and in a humid chamber. Irrespective of the storage condition, all microarrays could be used for at least two months without any apparent deterioration of the performance parameters. As a matter of fact, an increase of signal intensities was observed similar to the results seen with the initially blank slides.

3.7 Direct comparison of the different strategies

The four best performing slide surfaces: APTES-C4; MPTS-C4; GPTS and PL, were compared to three commercial slides: TeleChem aldehyde slides; nitrocellulose-coated FAST slides and epoxy-silanised QMT epoxy
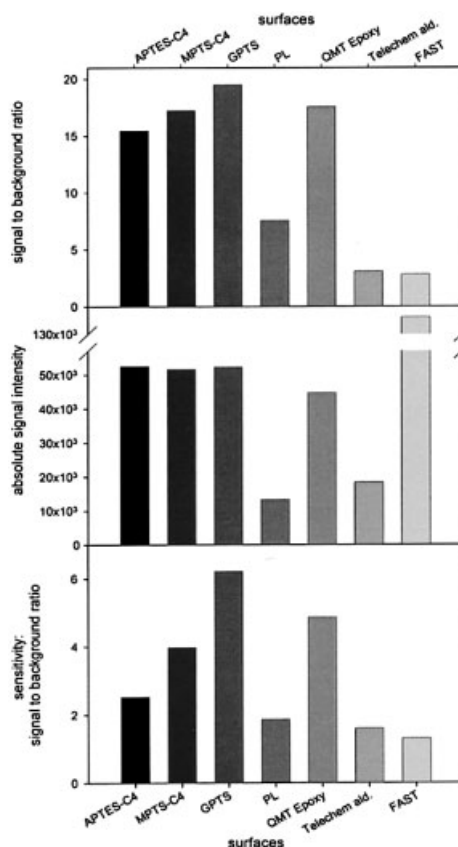

Figure 6. Direct comparison of various surfaces. All experiments were performed with an identical sample mixture at the conditions optimal to the respective system. The data presented here was calculated from the three signal intensities obtained on anti-KLH, antithydroglobulin and anti-γ-interferon. In the top panel, the signal-to-background ratios obtained at a total protein concentration of 100 μg/mL are shown. The central panel presents the means of the absolute signal intensities. Bottom panel: the sensitivity of detection was determined by incubations with the antigens at a concentration of around 10 pg/mL.

slides (Table 1). A protein mixture at a concentration of 100 μg/mL was labelled with Cy3 and applied to the various surfaces under the conditions that had been established as optimal for each individual system (Fig. 6). The overall best performance was observed with GPTS slides, although differences between APTES-C4 and MPTS-C4 as well as the commercial epoxysilane slides were relatively small. APTES-C4 and MPTS-C4 surfaces showed slightly higher background as GPTS and PL surfaces. Still, they out-performed PL slides by a factor of about three with respect to signal-to-background ratio.

Signal intensities on the nitrocellulose-coated FAST slides were the highest detected on either of the surfaces, being nearly three times as intense as the ones on the GPTS

slides. Unfortunately, however, the background is also very high, even after the application of a quenching reagent obtained from the manufacturer, rendering the advantage of strong absolute signals rather useless.

To test the assay sensitivity, we incubated the slides with 15 pg/mL KLH, 10 pg/mL thyroglobulin und 5 pg/mL γ-interferon. On all surfaces, these antigens could be detected, the specific signal not getting submerged in the background (Fig. 6). The relatively low signal-to-background ratios for APTES-C4 and PL slides were caused by high background on the former and low signal intensity on the latter surface. In separate experiments, even ten times lower antigen concentrations were still successfully detected on MPTS-C4 and GPTS slides, so that the sensitivity limit seems to be as low as about 1 pg/mL under the conditions used. This corresponds to a molar concentration of as little as 30 fM down to 0.25 fM, dependent on antigen size.

4 Discussion

The capacity of quantifying proteins and investigating on a global level their interactions and biochemical activities in a high-throughput manner could lead to a molecular characterisation of physiological or developmental cellular stages and will have a broad medical, diagnostic and commercial potential [1]. Protein microarray technology represents a promising tool in this respect. We studied, compared and optimised strategies for the production of antibody microarrays with the aim of creating a platform suitable to the various kinds of antigens present in a complex protein mixture.

The careful selection of an appropriate immobilisation procedure is a crucial step in developing such assays. In principle, there are four elementary processes, through which an antibody could be attached to a glass slide surface. Two – adsorption and binding *via* amino-groups – are common to all protein classes, and two – binding by thiol groups and carbohydrates – are rather specific to certain proteins such as antibodies. We tested these possibilities in quite a few variants. Procedures that required a modification of the antibodies prior to spotting were less suited to practical application. Similar difficulties were reported in other antibody immobilisation techniques; treatment with sodium periodate, for example, led to a loss of antibody activity of up to 20% [17], and subsequent purification steps could additionally cause losses of up to 50% of an antibody [16]. The production of microarrays, however, requires the handling of large numbers of expensive and sometimes rather unique protein molecules in small volumes. Too complex an attachment process is therefore difficult to manage and wasting valuable resources.

Antibody binding based on adsorption on aminosilane, poly-ʟ-lysine and FAST slides resulted in lower quality in comparison to the best performing surfaces. The sensitivity obtained corresponds to previously reported data [11, 18] and poly-ʟ-lysine and FAST slides are often used surfaces for protein microarray production [11, 12, 19, 24–26]. Nevertheless, relatively low signal intensities on the former and high background on the latter surface render them inferior to surfaces to which antibodies are attached by chemical coupling.

From the many options of antibody immobilisation on glass slides that we tested, the 2.5% epoxysilane (GPTS) surface performed best overall. It is simple to produce, showed very high sensitivity, very good signal-to-background ratios and good spot quality. The highly reactive epoxy surface is able to react not only with amino groups but also with other nucleophiles like alcohol, thiol and acid-groups on the protein surface [27]. Alternatively, mercaptosilane (MPTS) and aminosilane (APTES) surfaces with the relatively long maleinimido-NHS cross-linker C4 produced good results as well.

Optimal experimental conditions for the blocking process are mostly the result of empirical optimisation [28] and might require adaptation to specific preconditions. However, as known from other systems [*e.g.*, 29], too high a protein concentration always led on microarrays to a decrease in signal intensity. Small soluble proteins seemed to be more efficient reagents, while blocking with chemicals could badly affect the spot quality.

Also, the seemingly trivial aspect of spotting buffer composition had an influence on array quality, which could be improved significantly on GPTS, APTES and MPTS slides by the addition of trehalose in PBS. Its protective effect on proteins [30] for example in lyophilisation [31], freeze-drying [32] processes is well documented, the effectiveness apparently being dependent on the molecular ratio between protein and trehalose (at least 400:1, sugar:protein) rather than the additive's concentration [31].

Although identical cross-linkers were used, APTES-Cn and MPTS-Cn surfaces exhibited differences with respect to the binding of antigens of different sizes. Lacking correlation of cross-linker length and antibody activity on a mercaptosilane surface had also been reported earlier for immunosensors [16]. In addition, it is interesting that the APTES-C4 surface exhibited relatively high signal intensities, even without the additional step of antibody reduction prior to attachment. All this is most likely the result of an aligned and oriented antibody attachment on APTES-Cn surfaces. The existence of free thiol groups was reported for most antibody classes. Native serum IgGs, the biggest immunoglobulin fraction in a serum, contain 0.24 free thiol groups *per* immunoglobulin mole-

cule [33]. The level of free sulfhydryl-groups in several monoclonal IgG subclasses was determined with approximately 0.1 *per* IgG using detection with *N*-(1-pyrenyl) maleimide [34]. While β-barrel disulphides are mostly resistant to the reduction, the binding of maleimid groups may occur by reacting with the more accessible free thiol groups of the rather labile disulphides connecting the heavy and light chains [34, 35]. On the MPTS-Cn surfaces, sterical effects might be responsible for the bad binding, especially in the case of large antigens, since the length of the longest cross-linker tested (C4) is still relatively short with about 1.6 nm to permit proper exposure of randomly bound antibody molecules, which are of around 6 nm in size.

Since 90% of the mass of any proteome is contributed by approximately 10% of the proteins [3, 4, 36], analyses by antibody microarrays should be highly sensitive in order to detect the interesting proteins of low abundance. Despite the rather good sensitivity values reported here for the best attachment strategies, more development is needed in this direction. One important and challenging field is the labelling and detection of complex protein mixtures. Signal amplification by rolling circle amplification [37], tyramide amplification systems [38] or biotin-avidin/streptavidin based methods [13, 25, 39], for example, or the use of reporter molecules other than fluorescence, such as radioactive labelling, could be instrumental in improving assay sensitivity and accuracy further.

5 Concluding remarks

Although the results reported here seem to be more generally applicable, one should nevertheless be aware that particular antibodies or, even more likely, particular antigens, might not be amicable to certain surfaces, irrespective of their overall performance.

6 References

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