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Next generation of protein microarray support materials: Evaluation for protein and antibody microarray applications

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Abstract

The performance of protein and antibody microarrays is dependent on various factors, one of which is the use of an appropriate microarray surface for the immobilisation of either protein or antibody samples. We have investigated the properties of seven new surfaces in the context of both protein and antibody microarray technology. We have demonstrated the functionality of all new slide coatings and investigated the mean signal to spotted concentration ratio, determined detection limits and calculated coefficients of variation. Moreover, new concepts for slide coatings such as dendrimer and poly(ethylene glycol)-epoxy slides were evaluated and improved qualities of novel slide surfaces were observed. Optimal slide coatings for antibody and protein chips were proposed and the requirements for both technologies were discussed.

Keywords: Chip technology; Proteins; Antibodies

1. Introduction

Protein arrays are becoming increasingly important tools for protein interaction studies and diagnostics. Protein affinity assays have been used to analyse interaction between proteins such as antibodies, receptors or enzymes with other proteins, peptides, low-molecular mass compounds, oligosaccharides or DNA [1]. For improved performance, minimal reagent consumption and high-throughput screening, protein arrays have been miniaturised [2] and transferred to microscope glass slides [3]. This format allows rapid and inexpensive production in high quantities, detection by fluorescence with low autofluorescence, and ease of handling during storage and preparation procedures, as well as high reproducibility.

Most of the conventional microarray chip surfaces like poly-L-lysine coated slides have been adapted from DNA chip technology. Since the surface charge of proteins is variable, in contrast to DNA, which can simply be immobilised by electrostatic interactions of the phosphate backbone with a positively charged support material, further efforts have been undertaken to customise these materials for the more complex requirements of protein microarrays. For example, globular proteins in their native state usually display a hydrophilic exterior and a hydrophobic interior. Immobilisation to a hydrophobic surface may destabilise the structure and turn the

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inside out, thus rendering the protein inactive. However, profiling of an antibody's binding characteristics by such denatured protein microarrays is useful, since many antibodies will recognise linear epitopes of the denatured antigens. Antibody microarrays, in contrast, require the antibodies to remain active while presented on the surface in order to bind the labelled analyte specifically. Therefore, the immobilising material must preserve the active state of the antibody even during prolonged storage periods.

Other surface chemistries have been applied to achieve a homogeneous and specific retention of proteins and antibodies by covalent attachment and specialised surface groups. Several attempts have been made to reduce the destabilising surface contact by immobilisation via affinity tags [4] or by biotinylation of capture molecules and their immobilisation on streptavidin coated supports [5]. However, in high-throughput screening it is preferable to have a more generally applicable and single-step procedure by the use of optimised surfaces for protein immobilisation.

A new generation of surface chemistries has introduced surfaces that do not need any blocking reagent to reduce background binding and that prevent direct protein-surface contact by the introduction of a functionalised poly(ethylene glycol) (PEG) layer. Other chemistries optimise protein presentation by increasing the density of accessible functional groups using dendrimers [6,7]. However, both chemistries rely on the covalent coupling of proteins by epoxy groups. Here, we have compared these and other interesting materials to evaluate their performance in both protein and antibody microarray technology.

2. Material and methods

Monoclonal (Clone 1C8) anti-human serum albumin (anti-HSA) antibodies were purchased from DPC Biermann Diagnostika (Bad Nauheim, Germany), polyclonal anti-fibrinogen antibodies from Calbiochem-Novabiochem (San Diego, CA, USA) and Cy5-labelled goat anti-mouse IgG from Jackson ImmunoResearch Labs. (West Grove, PA, USA). Dilution rows of polyclonal anti-fibrinogen antibodies were prepared from 1.2 mg/ml to 750 ng/ml, dilution rows of human serum albumin (HSA) were prepared from 532 μg/ml to 330 ng/ml, both using 1×phosphate-buffered saline (PBS)/0.1% NaN_3. Human serum albumin and fibrinogen from human plasma were purchased from Sigma–Aldrich (Munich, Germany).

Fibrinogen was labelled with Cy3-NHS (Cy3-*N*-hydroxysuccinimide) dye (Amersham Biosciences, Freiburg, Germany) as recommended by the manufacturer. Unconjugated dye was removed using Ultrafree-0.5 centrifugal filters (Millipore, Bedford, MA, USA) with a molecular mass cut-off of 50 000.

Microarray slides were obtained from suppliers listed in Table 1. Polystyrene slides were activated by incubation in 0.1 *M* sodium hydrogen carbonate (pH 9.5) for 24 h. Poly-L-lysine slides were prepared as described in [8].

Dilution rows of anti-fibrinogen antibodies and HSA were spotted in two identical fields, each comprising twenty replicates of every dilution row (Fig. 1), using a QArray spotting robot (Genetix, Hampshire, UK) equipped with 150 μm solid pins. For spotting, a humidity of 65% was used for all slides except epoxy and PEG-epoxy slides, which were spotted at 30% humidity. After spotting, the slides were placed in a box at 4 °C overnight. The next morning, the slides were rinsed with TBS (10 m*M* Tris, 0.15 *M* NaCl, pH 7.4) and blocked in 3% (w/v) non-fat dry milk powder/TBS-T [TBS containing 0.1% (v/v) Tween-20] at 4 °C for 60 min. After rinsing again with TBS, the slides were incubated with labelled Cy3-fibrinogen at 4 μg/ml and monoclonal anti-HSA antibodies at 3.5 μg/ml at 4 °C for 60 min. The slides were rinsed with TBS and incubated with 1.3 μg/ml Cy5-labelled goat anti-mouse IgG at 4 °C for 60 min before being rinsed with TBS and washed twice in TBS-T at 4 °C for 15 min each. The slides were rinsed with TBS and centrifuged dry at 1000 *g* for 2 min. Scanning was performed using a ScanArray 4000 (Perkin-Elmer Life Sciences, Boston, MA, USA) using the same settings for all slides except FAST slides, which were scanned with a lower laser power and a lower gain. GenePix Pro 4.0 (Axon Instruments, Union City, CA, USA) software was used to analyse the scanned protein array images.

Table 1
Table listing the various slides used and their respective manufacturers

Name	Surface chemistry	Supplier
Prototypes		
Dendrimer slides	Dendrimer layer with reactive epoxy groups	Chimera Biotech GmbH, Dortmund, Germany www.chimera-biotec.de
PEG-Epoxy slides	PEG layer with reactive epoxy groups	Jens Sobek, Functional Genomics Center Zurich, Zurich, Switzerland/Microsynth GmbH, Schützenstrasse 15, 9436 Balgach, Switzerland jens.sobek@fgcz.unizh.ch
Commercially available		
Amine slides	Amine groups (extended chain length silane)	Telechem International, Sunnyvale, CA, USA
Epoxy slides	Epoxy groups	www.arrayit.com
Silanated slides	Amine groups	
FAST slides	Nitrocellulose-based matrix	Schleicher and Schuell Biosciences, Keene, NH, USA www.schleicher-schuell.com
Polystyrene cell culture slide	Polystyrene	Nalge Nunc International, Naperville, IL, USA www.nalgenunc.com

3. Results

Polyclonal anti-fibrinogen antibodies were evaluated for suitability and specificity in an antibody array format in [9]. Monoclonal anti-HSA was checked for functionality in enzyme-linked immunosorbent assay (ELISA) (data not shown).

After scanning, all slides were checked for proper spotting and aberrant spots were taken out of the analysis. Mean values of all replicates were calculated and a graph of mean signal intensity versus spotted concentration was generated displaying binding characteristics for each type of microarray support (Fig. 2). The average RSD for each slide coating was calculated by:

$$\mathrm{RSD} = \frac{\text{Standard deviation}}{\text{Mean}}$$

excluding signal intensities in the range of background fluorescence, to prevent a deviation of the coefficient due to random effects (Table 2).

The detection limit was defined as the lowest concentration at which the mean of signal intensities lay above the cut-off used in the calculation of the RSD (Fig. 3).

4. Discussion

In this paper, we investigated the performance of eight different coatings for the generation of protein and antibody microarrays. Plastic, as well as chemically modified glass slides were used. The use of microarrays for the study of antibody–protein interactions as well as protein–antibody interactions have been described in recent publications [2,5,8,10–13] and new concepts for the production of microarrays using an optimised environment have been published [14–16]. However, this study reveals novel microarray substrates of superior characteristics compared to microarray coatings of recent publications [9]. Additionally, we describe for the first time a microarray coating analysis, that compares microarray substrates for both of their main application areas, antibody and protein microarray technology simultaneously. In contrast to proteins, antibodies have to maintain

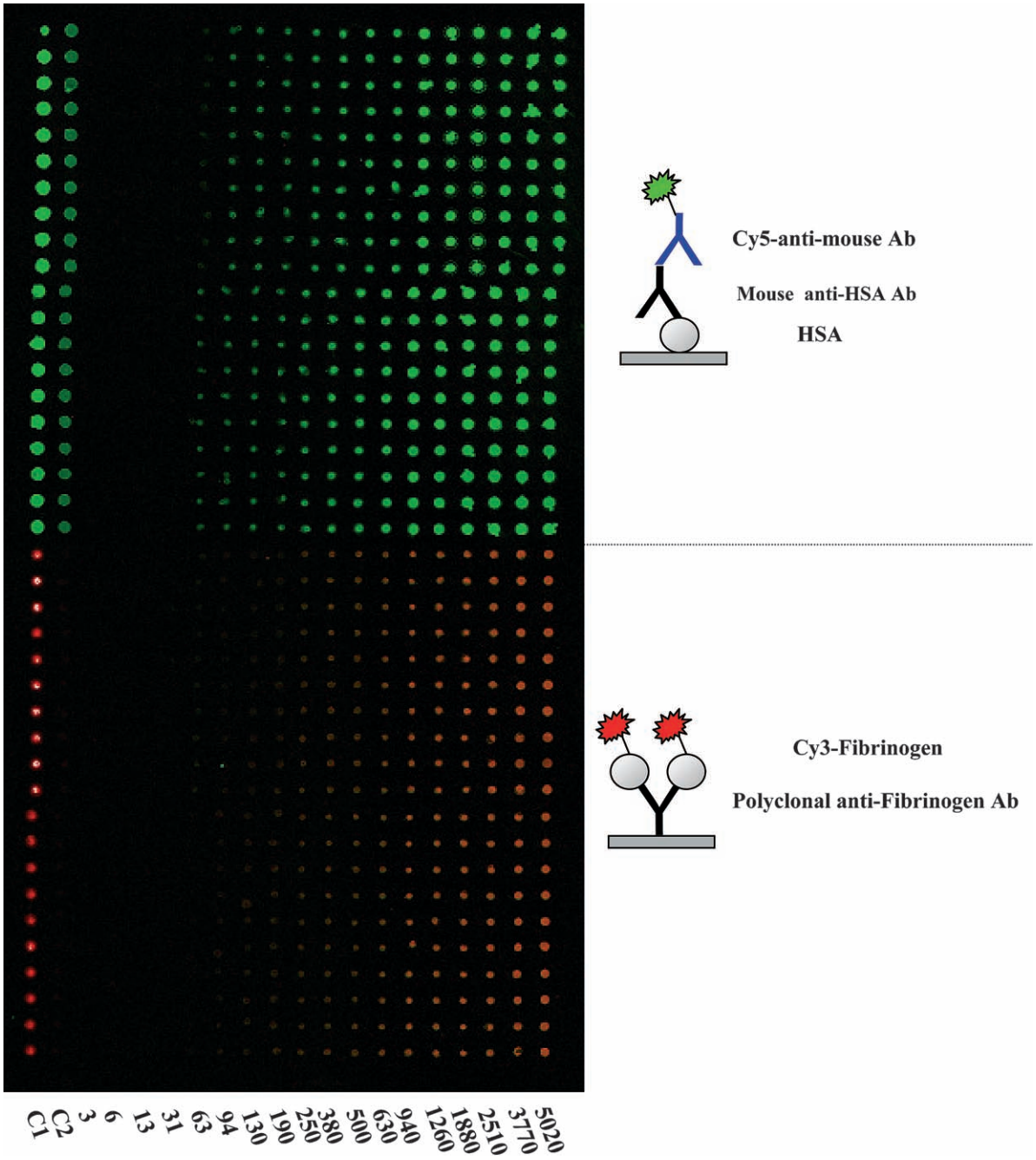

Fig. 1. Scan of a microarray consisting of a protein microarray at the top (green spots) and an antibody microarray at the bottom (red spots). The schematic assembly of all components is indicated next to the scan. The absolute amounts of the immobilized binder are indicated in attomoles of protein or antibody per spot, for each column. C1 and C2 represent controls of the fluorescent labelling, in which the labelled component was immobilised directly. For the protein microarray, concentrations of 130 μg/ml (C1) and 13 μg/ml (C2) of Cy5-anti-mouse IgG were immobilised. For the antibody microarray, concentrations of 400 μg/ml (C1) and 40 μg/ml (C2) of Cy3-fibrinogen were immobilised.

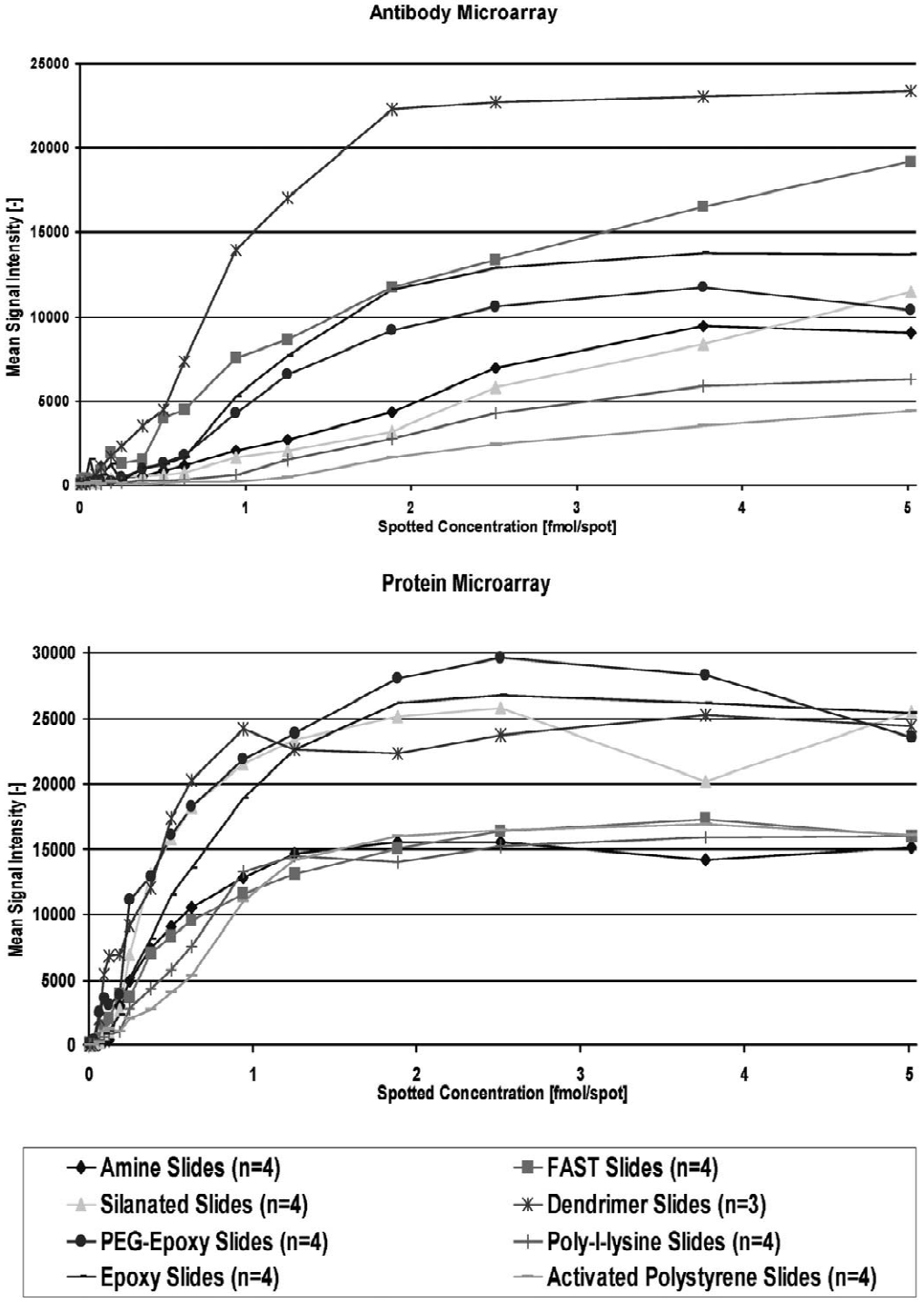

Fig. 2. Graphs showing mean signal intensities (relative units) versus absolute amounts of spotted binder per spot for eight different microarray materials. Each point represents the mean signal intensity of all spots at a specific concentration. The number of tested chips (*n*) is indicated in the graph.

Table 2
RSDs

	RSD (%)		
	Antibody chips	Protein chips	Average
Dendrimer slides	24	21	22
PEG Epoxy slides	24	25	24
Poly-L-lysine slides	35	16	26
Amine slides	41	21	31
FAST slides	32	30	31
Activated polystyrene slides	41	26	33
Epoxy slides	43	24	34
Silanated slides	35	40	38

native confirmation of antigen binding regions to remain functional. Microarray coatings that show good characteristics with regard to their use in antibody microarray technology may, therefore, have potential to be applied in analysis of more complex protein–protein interactions, in which native conformation of the immobilised binding partner is crucial.

Investigation of substrates for protein microarray technology reveals two groups of surfaces, which display similar signal intensity versus spotted concentration relationship. The one with higher signal intensities comprises both, covalently binding surfaces, such as PEG-epoxy slides, epoxy slides and dendrimer slides as well as non-covalently binding surfaces like silanated slides. The group with generally lower signal intensities comprises only non-covalently binding surfaces, which bind proteins by electrostatic or hydrophobic interactions, such as amine slides, poly-L-lysine slides and activated polystyrene slides. Although FAST slides are also both, a member of this group and a non-covalently binding surface, it is difficult to compare this slide, since it was scanned with a lower gain, to avoid excessive background fluorescence. Nevertheless, all tested surfaces demonstrate a saturation of mean signal intensity in the region of 2000–2500 amol/spot. Dendrimer coated slides show an even earlier saturation of signal in the range of 940 amol/spot. This shifts the dynamic range, in which a quantitative measurement in future applications may be possible, to concentrations below 2000 amol/spot.

Detection limits of all slides with respect to their use in protein microarray technology allows a separation of all surfaces in three groups. Relatively high detection limits were observed with activated polystyrene slides, with detection limits of about 190 amol/spot. Relatively low detection limits were obtained with amine slides and dendrimer slides with a detection down to 63 amol/spot. All other slides display similar detection limits of about 94 amol/spot.

Investigation of the signal intensity versus concentration relationship for antibody microarray coatings did not reveal distinct groups as with protein microarrays. Most coatings demonstrated a linear relationship between signal intensity and concentration and only dendrimer slides displayed a definite saturation of signal intensity above 1880 amol/spot. Few surfaces, such as epoxy, PEG-epoxy and amine slides display saturation above a concentration of 3770 amol/spot.

Detection limits of antibody microarrays are, similar to the performance in the signal versus concentration diagram, rather diverse. A grouping of detection limits as done with protein microarrays is not possible. However, similar results were obtained in respect to activated polystyrene, which display very high detection limits, whereas amine, as well as dendrimer slides, show very low detection limits. Generally, detection limits of antibody microarrays are higher in comparison to protein microarrays.

Average coefficients of variation vary between 22% and 38%. Relatively low variations are obtained with dendrimer and PEG-epoxy slides, while silanated slides display rather high RSDs. RSDs of antibody microarrays are higher in comparison to protein microarrays. Only dendrimer, epoxy, FAST

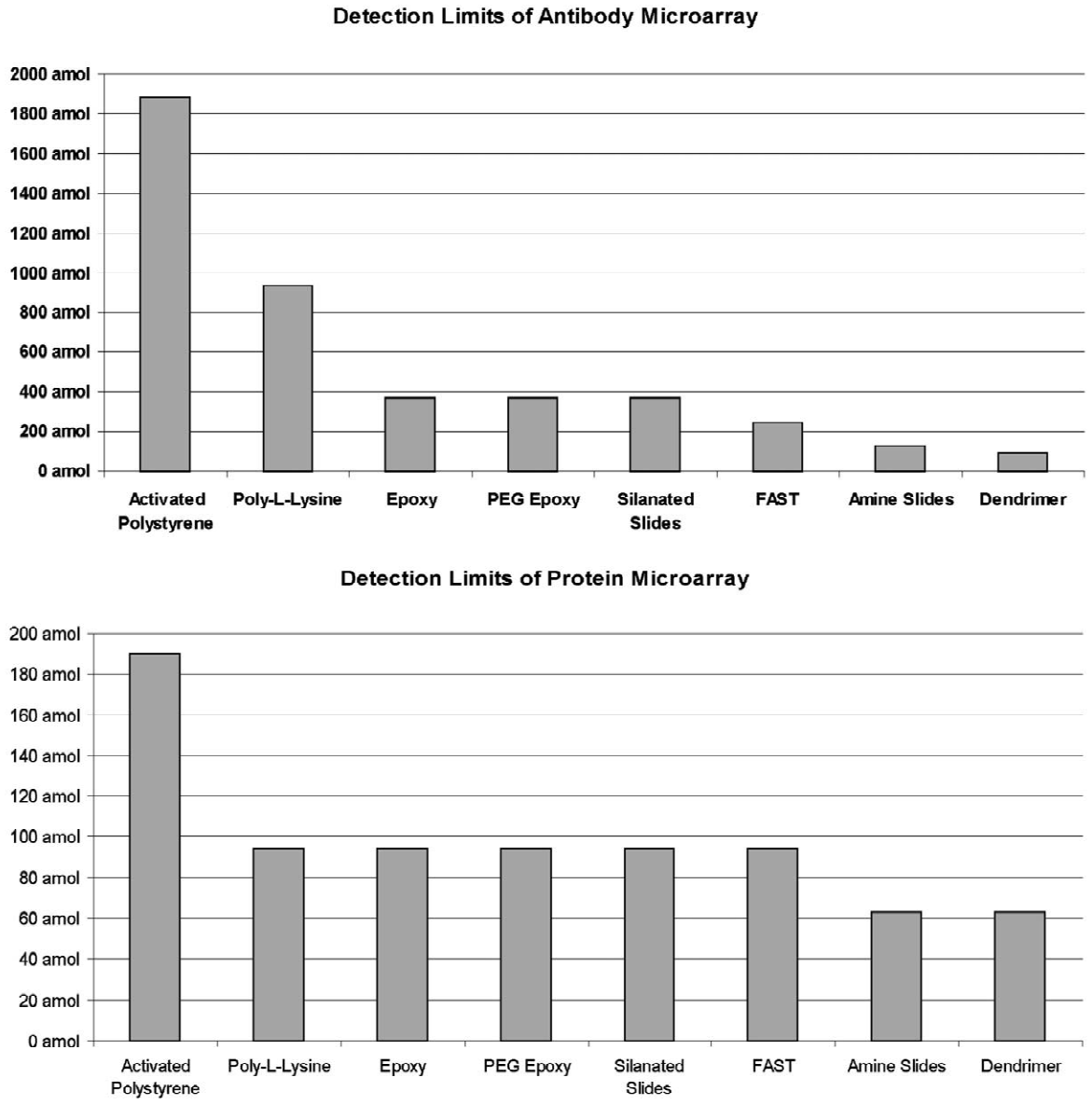

Fig. 3. Diagram displaying the detection limits of eight different surface materials for antibody and protein microarrays, which was defined as least concentration step at which the mean of signal intensities lay above the cut-off used in the calculation of the coefficient of variation.

and silanated slides do not show large deviations of the mean coefficient of variation between both types of applications.

In order to compare results gained in this study to results from [9], in which a similar evaluation was performed, poly-L-lysine slides were tested in both. Calculations of the amount of antibodies and proteins were adjusted on recent findings, which suggest that the amount of solution transferred to the chip is 0.62 nl [17] and not 5 nl as presumed in [9]. To maintain comparability between both studies, the same spotting machinery, protocols and buffers were used instead of different optimised handling procedures supplied by the manufacturers. However, a different

performance of poly-L-lysine slides with regard to detection limit and coefficients of variation were obtained in this study. This demonstrates the difficulty of protein microarray technology to obtain identical signal intensities on repetition of experiments. Reasons for that are differing efficiencies in fluorescent labelling, as well as differences in quality between different batches of manually coated poly-L-lysine slides. This necessitates the use of identical reagents as well as the consecutive handling of different surfaces within a comparison study. Therefore, poly-L-lysine slides were evaluated in both studies to allow a relative comparison. The fact that poly-L-lysine slides show the second highest detection limits on antibody microarrays as well as relatively low signal to concentration relationship suggests that most surfaces presented in this study are superior in comparison to [9].

Although it is difficult to predict suitability of microarray coatings for protein and antibody microarray technology using one protein and one antibody, the results presented here point towards coatings that offer outstanding qualities in their respective area of application. As expected, one surface for both antibody and protein microarray applications could not be found. For quantitative measurements of proteins in complex samples, dendrimer slides show an excellent signal to concentration ratio. For the quantitative measurement of antibodies, PEG-epoxy slides are the surface coating of choice. For experiments that necessitate the detection of very low abundance proteins and antibodies, both, dendrimer slides as well as amine slides are very well suited. Nevertheless, FAST slides also display very good detection limits in antibody microarray technology.

In conclusion, we have tested eight different microarray coatings for the routine use of protein and antibody microarray technology simultaneously. We demonstrated the functionality of all new slide coatings and investigated the mean signal to spotted concentration ratio as well as the detection limits and the coefficients of variation. Moreover, new concepts for slide coatings such as dendrimer and PEG-epoxy slides were introduced and improved qualities of most novel slide surfaces in comparison to previously published ones were shown. Optimal slide coatings for antibody and protein chips were proposed and the requirements of both technologies were discussed.

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