

RESEARCH ARTICLE

Design of recombinant antibody microarrays for complex proteome analysis: Choice of sample labeling-tag and solid support

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Antibody-based microarray is a novel technology with great potential within high-throughput proteomics. The process of designing high-performing antibody (protein) microarrays has, however, turned out to be a challenging process. In this study, we have developed further our human recombinant single-chain variable-fragment (scFv) antibody microarray methodology by addressing two crucial technological issues, choice of sample labeling-tag and solid support. We examined the performance of a range of dyes in a one- or two-color approach on a selection of solid supports providing different surface and coupling chemistries, and surface structures. The set-ups were evaluated in terms of sensitivity, specificity, and selectivity. The results showed that a one-color approach, based on NHS-biotin (or ULS-biotin) labeling, on black polymer Maxisorb slides (or Netherion slide H) was the superior approach for targeting low-abundant (pg/mL) analytes in nonfractionated, complex proteomes, such as human serum or crude cell supernatants. Notably, microarrays displaying adequate spot morphologies, high S/Ns, minimized nonspecific binding, and most importantly a high selectivity, specificity, and sensitivity ($\geq$ fM range) were obtained. Taken together, we have designed the first generation of a high-performing recombinant scFv antibody microarray technology platform on black polymer Maxisorb slides for sensitive profiling of low-abundant analytes in nonfractionated biotinylated complex proteomes.

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1 Introduction

In recent years, high-throughput genomics has provided the blueprints for numerous genomes, paving the way

for major breakthroughs in biomedicine [1–3]. Inspired by these advances, proteomics – the large scale analysis of proteins – has become a key discipline for addressing protein expression patterns and for understanding function and regulation of entire set of proteins encoded by an organism [2, 4–7]. In the end, such information will be critical for understanding complex biological processes at the molecular level, how they differ in various tissues and cell types, and how they are affected in disease states [2, 8].

Turning to the proteome, there is an exponential increase in biological complexity placing high demands on the technologies applied [2, 9, 10]. In fact, there are very few effective

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Abbreviations: CT, cholera toxin subunit B; FB, factor B; IL, interleukin; MoDC, monocyte-derived dendritic cell; NHS, N-hydroxysulfosuccinimide ester group; scFv, single-chain variable-fragment; ULS, universal linkage system

strategies for high-throughput profiling of entire proteomes [2]. In traditional proteomics, various separation methods, such as 2-D gels, coupled with MS have turned out to be a versatile proteomic research tool frequently used [11–14]. Although this is a powerful approach for proteome analysis, it is partially impaired by a limited resolution (<2000 protein analytes *per* sample), low-throughput, and limited sensitivity [10, 13]. Thus, the need for rapid and multiplexed proteomic approaches displaying high specificity, selectivity, and sensitivity is considerable.

Antibody-based microarrays are among the novel class of rapidly evolving technologies with great potential within high-throughput (disease) proteomics [2, 9, 15–17]. Miniaturized microarrays (<1 cm²) can be printed with numerous individual antibodies, and while consuming only minute amount (μL scale) of reagents, rapid, sensitive, and selective assays can be performed [2, 9, 17–19]. In recent years, the first generation of antibody microarrays, based mainly on intact monoclonal and polyclonal antibodies, have emerged [9, 16–18, 20–23], clearly outlining the potential of the technology [24–27]. Despite the success, these efforts have also demonstrated that several key technological issues remains to be addressed before high-performing antibody-based microarrays for global proteome analysis targeting low-abundant analytes in complete, *i.e.*, nonfractionated, proteomes can be developed and made available [9, 17, 28].

We have focused our efforts on developing recombinant antibody microarrays for high-throughput proteomics [29–34]. To this end, we have used human recombinant single-chain variable-fragment (scFv) antibody fragments, selected from a large (2×10^{10} members) phage display library [35] as probes. Initially, we have addressed several of the basic microarray technological features, such as design, format, and immobilization of the probes [33–36], design of the solid support [30], sample format [29], array design [37, 38], and assay sensitivity [33]. To date, we have successfully developed the first recombinant scFv antibody microarrays for multiplexed and sensitive analysis of semicomplex proteomes [9, 33].

In this study, we have developed further our recombinant antibody microarray design by addressing two remaining critical key technological issues, choice of sample labeling-tag and solid support. To this end, we examined our ability to target low-abundant analytes (pg/mL), *e.g.*, cytokines, in complex proteomes, such as crude serum or cell expression supernatants. The samples were labeled with a range of dyes in a single- or dual-color read-out mode and analyzed on microarrays fabricated on a set of solid supports displaying different physico-chemical properties. The results showed that we have successfully designed the first generation of a high performing recombinant scFv antibody microarray technology platform on black polymer Maxisorb slides for sensitive (pM to fM range) detection of low-abundant protein analytes in nonfractionated biotinylated complex proteomes.

2 Materials and methods

2.1 Antibodies

Twenty human recombinant scFv antibody fragments directed against ten low-abundant serum protein analytes (chemokines, cytokines, and growth factors) (pM to fM range), including interleukin (IL)-1α (clone IL-1α-145 and IL-1α-122), IL-4 (clone IL-4-55), IL-6 (clone IL-6-64), IL-8 (clone IL-8-39), IL-12 (clone IL-12-38), IL-16 (clone IL-16-4), IL-18 (clone IL-18-14), MCP-4 (clone MCP-4-8), TNF-β (clone TNF-β-19), VEGF (clone VEGF-3), and eight medium abundant serum analytes (complement factors) (μM to nM range in serum), including C1q (clone C1q-4), C1s (clone C1s-8), C3 (clone C3-7), C4 (clone C4-3), C5 (clone C5-12), C1 esterase inhibitor (C1-INH) (clone EI-12), factor B (FB) (clone FB-7), and properdin (clone Prop-3), as well as 1 nonserum protein analyte, cholera toxin subunit B (CT) (clone CT-17), were selected from the n-CoDeR library [35] and kindly provided by BioInvent International AB (Lund, Sweden).

All scFv antibodies were produced in *E. coli* and purified by affinity chromatography on Ni-NTA agarose (Qiagen, Hilden, Germany). Bound molecules were eluted with 250 mM imidazole, extensively dialyzed against PBS, concentrated and stored at 4°C until further use. The protein concentration (average concentration 165 μg/mL) was determined using a Micro BCA™ Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). The integrity and purity of the scFv antibodies were confirmed by 10% SDS-PAGE (Invitrogen, Carlsbad, CA, USA).

2.2 Antigens

Pure human recombinant IL-1α, IL-6, IL-8, TNF-β, and VEGF were purchased from R&D (Flanders, NJ, USA). Purified human C1q, C3, C5, FB, and properdin were purchased from Quidel (San Diego, CA, USA). Purified and biotinylated CT were obtained from Sigma (St. Louis, MO, USA).

Human whole serum or cell supernatants from activated cells were used as complex proteomes. Normal human serum was obtained from healthy blood donors (Lund University Hospital, Lund, Sweden). The cell supernatants were generated from stimulated human monocyte-derived dendritic cells (MoDCs). Briefly, human monocytes were purified from leukocyte-enriched buffy coats (Lund University Hospital), as previously described [39]. Briefly, the cells were >99.5% CD14⁺ as determined by flow cytometry. To generate MoDCs, CD14⁺ cells were cultured (5×10^5 cells/mL) for 7 days in R10 complete medium supplemented with rhGM-CSF (300 ng/mL) (Leukomax; Sandoz, Schering-Plough, Innishannon, Ireland) and rhIL-4 (100 ng/mL) (R&D Systems, Minneapolis, MN, USA) [39]. Next, the MoDCs were stimulated for 24 h with 25% v/v monocyte conditioned medium [39],

rhIL-1 β (10 ng/mL) (R&D Systems), and rhTNF- α (10 ng/mL) (R&D Systems), whereafter the cell supernatants were harvested and immediately frozen (-80°C) until further use. The MoDC phenotype was monitored by following the expression of the cell surface molecules CD80, CD86, and HLA-DR, using FACS analysis. The complexity of the retrieved cell supernatants was mainly determined by the 10% v/v FBS included in the cell culturing medium.

2.3 *N*-hydroxysulfosuccinimide ester group (NHS)-biotin labeling of samples

The samples were biotinylated using the EZ-Link Sulfo-NHS-LC-Biotin (Pierce). Briefly, serum (diluted 45:1) or cell supernatants were diluted in PBS, resulting in a concentration of about 2 mg/mL. The protein concentration was determined using a Micro BCA Protein Assay Reagent Kit (Pierce). The samples were labeled at a molar ratio of biotin/protein of 45:1, assuming an average molecular weight of 150 kDa for the proteins. In one experiment using serum as sample, the molar ratio of biotin to protein was titrated, ranging from 2:1, 5:1, 15:1, 45:1, 90:1, 180:1, and 450:1. The biotinylation reactions were initiated by the addition of EZ-Link Sulfo-NHS-LC-Biotin to the diluted samples. The reactions were allowed to continue for 2 h on ice, with gentle vortexing every 20th minute. Unreacted biotin was removed by extensive dialysis against PBS for 72 h using a 3.5 kDa MWCO dialysis membrane (Spectrum Laboratories, Rancho Dominguez, CA, USA). The samples were aliquoted and stored at -20°C until further use. Pure analytes (IL-1 α , IL-6, IL-8, and IL-12) were labeled in a similar manner, but at a molar ratio of 10:1 (dye/protein).

2.4 Alexa-647 and Alexa-555 labeling of samples

The samples were labeled with Alexa-647 and/or Alexa-555 according to the instructions provided by the supplier (Molecular Probes, Eugene, OR, USA). Briefly, serum (diluted 45:1) or cell supernatants were dialyzed against and/or diluted in labeling buffer labeling buffer (0.1 M Na_2CO_3 , pH 8.3) to a concentration of 2 mg/mL. The protein concentration was determined using a Micro BCA Protein Assay Reagent Kit (Pierce). The samples were labeled at a ratio of 1:5 (mg dye:mg protein) for 1 h at room temperature and the mixtures were gently mixed every 10 min. Finally, the samples were extensively dialyzed against PBS at 4°C and stored at -20°C until use. Pure analytes (TNF- β , IL-1 α , IL-6, IL-8, and IL-12) were labeled in a similar manner, but at a molar ratio of 10:1 (dye/protein).

2.5 Cy-3 and Cy-5 labeling of samples

The samples were labeled with Cy-3 and/or Cy-5 mono-reactive dye following the recommendations of the manu-

facturer (GE Healthcare, Piscataway, NJ, USA). Briefly, serum (diluted 45:1) or cell supernatants were dialyzed against and/or diluted in labeling buffer labeling buffer (0.1 M sodium carbonate, pH 9.3) to a concentration of 2 mg/mL. The protein concentration was determined using a Micro BCA Protein Assay Reagent Kit (Pierce). The samples were labeled at a ratio of mg protein to mg dye of 0.2 for 1 h at room temperature. Finally, the samples were extensively dialyzed against PBS at 4°C and stored at -20°C until use. Pure CT was labeled in a similar manner, but at a ratio of 1 for 0.5 h at room temperature [33]. Pure C1q was labeled at a molar ratio of 0.5 for 3 h at room temperature as previously described [33].

2.6 Universal linkage system (ULS)-labeling of samples

The samples were labeled with ULS-biotin and/or ULS-flu according to the instructions provided by the supplier (Kretech, Amsterdam, Netherlands). Briefly, the samples were diluted 1:50 in H_2O (to a concentration of about 1.8 mg/mL) and incubated with ULS-biotin or ULS-flu (diluted 1:20) for 16 h at 37°C . Unreacted dye was removed by affinity purification using ULS-trap columns. The ULS-labeled samples were immediately analyzed on the recombinant antibody microarrays.

2.7 Solid supports

Seven well-performing solid supports, providing different surface structure/surface chemistry/coupling chemistry, were selected and evaluated. Planar silicon (planar/silicon surface/adsorption; Addison Engineering, San Jose, CA, USA), Xenoslide N (planar/ Ni^{2+} -chelate derivatized/affinity binding; Xenopore, Hawthorne, NJ, USA), FAST-slides (3-D surface/NC coated/adsorption; old vs. new formula) (Whatman, Florham Park, NJ, USA), Nexterion slide H (3-D surface/hydrogel/covalent binding; Schott-Nexterion, Mainz, Germany), protein binding microarray slide (planar/proprietary information/adsorption; NUNC A/S, Roskilde, Denmark), and black polymer Maxisorb (planar/proprietary information/adsorption; NUNC A/S).

2.8 Production and processing of scFv antibody microarrays

Each scFv were dispensed in eight replicates onto the solid support, using a noncontact Biochip Arrayer1 (Perkin Elmer Life & Analytical Sciences, Wellesley, MA, USA). The scFvs were arrayed by spotting three drops (333 pL/drop) on top of each other at each position (the spots were allowed to dry out in between). A hydrophobic pen (DakoCytomation Pen, DakoCytomation, Glostrup, Denmark) was used to draw a hydrophobic barrier around the arrays.

Next, the arrays were blocked with blocking solution for 1 h. All incubations were performed in a humidity chamber at room temperature for 1 h. Numerous blocking solutions were evaluated, including 1–5% w/v fat-free milk powder (Semper AB, Sundbyberg, Sweden) in PBS or TBS, 1–5% w/v BSA (Sigma) in PBS or TBS, 1–5% ovalbumin (Sigma) in PBS or TBS, 1–5% w/v casein (Sigma) in PBS, 1–5% w/v PEG2000 (Sigma) in PBS, 0.5–2% v/v Triton X-100 (Merck, Whitehouse station, NJ, USA), 0.5–2% v/v Tween-20 (Merck) in PBS or TBS, 0.5–2% v/v NP-40 (Sigma) in PBS, 0.5–2% w/v *N*-octyl- β -D-glucopyranoside (Sigma) in PBS, 0.5–2% w/v *N*-octanoyl-*N*-methylglucosamin (Sigma) in PBS, 0.5–2% w/v PVP-40 (Sigma) in PBS, and 0.1–0.2 mg/mL PLL-g-PEG (SurfaceSolutionS, Switzerland) (molecular weight $\sim$ 68 kDa) in 10 mM Hepes, pH 7.4. Subsequently, the arrays were washed four times with 60 μ L 0.05% v/v Tween-20 in PBS (PBS-T) or blocking solution (diluted 1–20 times in PBS). Next, 60 μ L of the labeled sample, diluted (1–100 times) in PBS or blocking solution (diluted 1–10 times in 0.5–2% v/v Tween-20 PBS) (sample buffer) was added and the chip was incubated. The arrays were washed four times with 60 μ L PBS-T and, in the case of biotinylated samples subsequently incubated with 60 μ L, 1 μ g/mL, Alexa-647 labeled streptavidin diluted in sample buffer. Finally, the arrays were washed four times with PBS-T and then dried out under a flow of nitrogen gas and immediately scanned with a confocal microarray scanner (Scan Array Express, Perkin Elmer Life & Analytical Sciences) at a resolution of 5 μ m using six different scanner settings. The ScanArray Express software V2.0 (Perkin Elmer Life & Analytical Sciences) was used to quantitate the intensity of each spot using the fixed circle method. The local background was subtracted as was negative control (nonspecific scFv) and each data point represents the mean value of the eight replicates.

2.9 Specificity and sensitivity of scFv antibody microarrays

To confirm the specificity of the arrayed scFvs, spiking experiments were carried out. In this case, biotinylated (45:1) serum was spiked with pure unlabeled (10 pg/mL) or labeled (100 pg/mL) cytokines (TNF- β , IL-1 α , IL-6, IL-8, IL-12, or VEGF) prior to the antibody microarray analysis. The signal intensities were compared with those obtained for the unspiked sample.

The LOD was measured by analyzing serial dilutions of pure analytes and/or labeled complex serum/cell expression supernatant samples. The detection limit was defined as the signal intensity of the negative control plus 2 SDs. A two-tailed *t*-test was used to determine whether the observed signals were significantly above the LOD ($p < 0.05$).

3 Results

In this study, we have developed further our recombinant scFv antibody microarray design to target low-concentrated protein analytes (pg/mL) in nonfractionated crude proteomes, such as serum or cell expression supernatants. To this end, we have addressed two of the key technological microarray parameters, including choice of sample labeling-tag and substrate. A variety of dyes were evaluated in a single- or dual-color read-out mode on microarrays fabricated on a set of seven solid supports displaying different physico-chemical properties.

3.1 One- or two-color labeling approach

First, we examined the influence of adopting a one- or two-color labeling approach for complex proteome analysis using recombinant antibody microarrays. Crude human serum and cell expression supernatants from activated MoDCs were individually labeled with Cy3, Cy5, Alexa-647, Alexa-555, ULS-biotin, ULS-flu, or NHS-biotin. Subsequently, the samples were analyzed on microarrays fabricated on a set of six solid supports, including planar silicon, Xenoslide N, FAST-slides (old and new formula), protein binding slides and Maxisorb slides, and the signal intensities for the matching pairs of dyes were compared. The analysis were performed both as two-color assays, *i.e.*, the matching labeling of each sample were mixed at equivalent amounts and analyzed on the same array, and as one-color assays, *i.e.*, analyzed on separate arrays. Representative results, illustrated by those obtained for Cy3/Cy5 on Xenoslide N (Fig. 1A), Alexa-647/Alexa-555 on Xenoslide N (Fig. 1B), ULS-biotin/ULS-flu on FAST-slides (new formula) (Fig. 1C), and ULS-biotin/ULS-flu/NHS-biotin on Maxisorb slides (Fig. 1D) are shown in Fig. 1.

The results showed that a distinct discrepancy in the signal intensities was observed for the two dyes in each matching pair (Fig. 1). Similar results were obtained whether the assays were run as two- or one-color assays, and irrespective of the solid support used (data not shown). In more detail, the average signal intensity ratio of Cy5: Cy3 was found to be 4 (Fig. 1A), 3 for Alexa-647: Alexa-555 (Fig. 1B), and 6 for ULS-biotin: ULS-flu (Figs. 1C and D). These skewed signal intensity ratios were accompanied with an average 3–7 times improved S/N for the best performing dye (Cy5, Alexa-647, and ULS-biotin) in each pair. Similar results were observed analyzing serial dilutions (1:1–1:100) of the samples (data not shown). Of note, very low signal intensities were observed for the least performing dye (Cy3, Alexa-555 and ULS-flu) in each pair (Fig. 1), making a two-color approach less favorable. Further, NHS-biotin was found to perform equally well, or better, than ULS-biotin with respect to the observed signal intensities (Fig. 1D). Hence, the results implied that a one-color approach (Cy5, Alexa-647, ULS-biotin, or NHS-biotin) should be adopted to obtain high and adequate signal intensities when targeting complex samples.

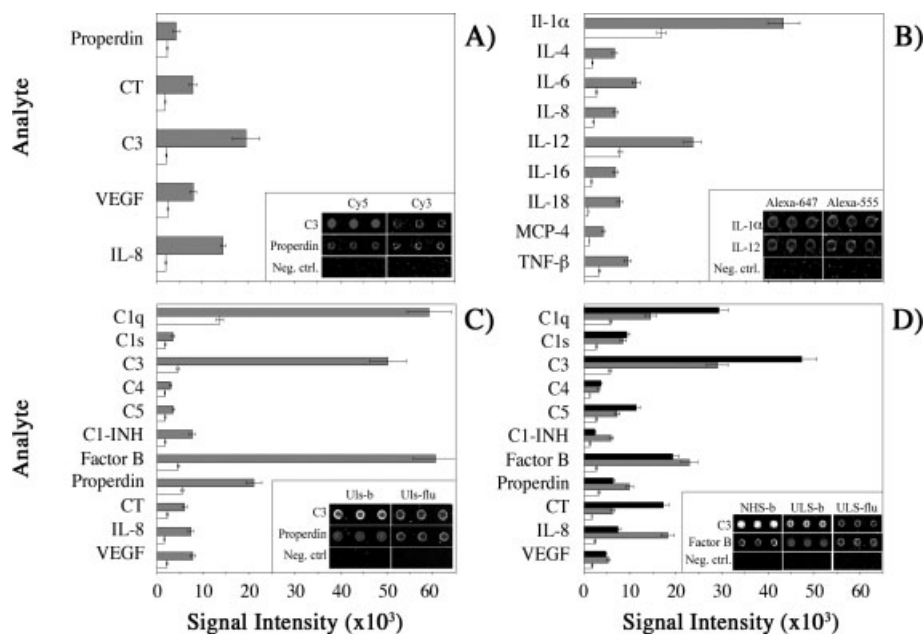


Figure 1. Comparison of one- and two-color labeling of complex proteomes for recombinant antibody microarray analysis performed on various solid supports. (A) Cy5 (gray bars) and Cy3 (open bars) labeled human serum analyzed on Xenoslide N slides. (B) Alexa-647 (gray bars) and Alexa-555 (open bars) labeled cell expression supernatants analyzed on Xenoslide N slides. (C) ULS-biotin (gray bars) and ULS-flu (open bars) labeled human serum analyzed on FAST slides (new formula). (D) NHS-biotin (black bars), ULS-biotin (gray bars), and ULS-flu (open bars) labeled human serum analyzed on black polymer Maxisorb slides. Microarray images from a selected set of analytes are shown. The array signal intensities shown represent the mean value of eight replicates after subtracting local background and negative control, *i.e.*, meaning that the signals should reflect specific signals. Similar results were obtained whether the analysis was performed as two-color assays or one-color assays. Representative data from one out of three or more experiments are shown. NHS-b = NHS-biotin, ULS-b = ULS-biotin, Neg. ctrl. = negative control.

3.2 LOD

Next, we examined the influence of the choice of sample labeling-tag and solid support on the LOD (Table 1). To this end, three of four top dyes identified in Fig. 1, including Cy5, Alexa-647, and NHS-biotin, were selected and analyzed on recombinant antibody microarrays fabricated on five different substrates. Adopting a single-color approach, the LOD was then determined for a selection of low-abundant analytes (IL-1 α , IL-6, IL-8, IL-12, and TNF- β), one medium-abundant analyte (C1q), and one (spiked) reference protein (CT). Similar results were obtained whether the analytes were targeted in pure format or (spiked) in crude human serum or cell expression supernatants (data not shown).

The results showed that the LOD was improved 2–18 times by labeling the analytes with NHS-biotin than with Cy5 (C1q and CT) (Table 1). Similarly, the LOD was enhanced 60–2000 times by tagging the proteins with NHS-biotin rather than with Alexa-647 (IL-8 and IL-12). Hence, the results demonstrated that the best LOD values were obtained using NHS-biotin as sample labeling-tag.

Moreover, the LODs were also found to be highly dependent on the solid support adopted (Table 1). Based on the LODs, the substrates could be ranked in the order of Maxisorb > protein binding slide > Fast-slide and Xenoslide

N > planar silicon. In fact, the LOD was shown to be improved 40–1000 times by using the highest ranking support, Maxisorb slides, than the lowest ranking substrate, planar silicon slides. Notably, LODs in the pM to sub-fM range were observed. Taken together, the results showed that the best LODs were obtained by analyzing NHS-biotinylated analytes on recombinant antibody microarrays fabricated on black polymer Maxisorb slides.

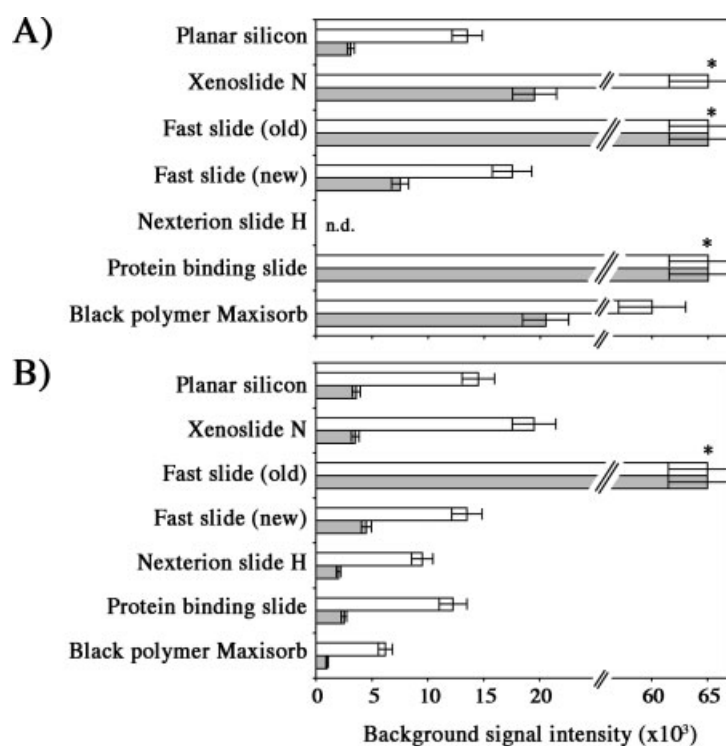
3.3 Choice of solid support – nonspecific background binding

To investigate the choice of solid support in more detail, we examined the nonspecific background binding, *i.e.*, how well the solid supports could be blocked, and the impact of this on our ability to detect high- and low-abundant protein analytes in complex proteomes (Fig. 2). Human serum and cell expression supernatants were labeled with two of the best performing single dyes, Alexa-647 and NHS-biotin, and analyzed on microarrays produced on seven (including two variants of FAST-slides) different solid supports. Representative results, illustrated by those observed for Alexa-647 (Fig. 2A) and NHS-biotinylated (Figs. 2B and C) serum samples are shown in Fig. 2.

Table 1. Influence of the choice sample labeling-tag and solid support on the LOD for recombinant antibody microarrays. Similar results were obtained whether the protein analytes were targeted in pure format or in crude human sera and cell expression supernatants

Solid support	LOD (pM)									
	TNF- β		IL-1 α		IL-6		IL-8		IL-12	
	A-647	A-647	A-647	Biotin	A-647	Biotin	A-647	Biotin	C1q	CT
Planar silicon	1500	300	1600	–	600	–	40	–	65	2000
Xenoslide N	–	–	–	–	600	–	–	–	6.5	1000
FAST slide ^{a)}	–	–	16	–	60	–	–	–	6.5	1000
Protein binding slide	–	–	–	–	6	–	–	–	6.5	42
Black polymer Maxisorb	1.5	3	1.6	0.003	6	0.0006	0.04	0.3	0.65	14

a) Old formula.

**Figure 2.** Influence of the choice of sample labeling-tag and solid support on the non-specific background signals and detection of low- and high-abundant protein analytes in complex proteomes. (A) Non-specific background signals for Alexa-647 labeled human serum analyzed on a set of six solid supports displaying different physico-chemical properties (see Section 2) scanned at two scanner settings (open and gray bars). (B) Nonspecific background signals for NHS-biotinylated human serum analyzed on a set of six solid supports displaying different physico-chemical properties scanned at two scanner settings (open and gray bars). (C) Detection of low- and medium-abundant protein analytes in NHS-biotinylated human serum analyzed on a set of six solid supports displaying different physico-chemical properties. The mean relative S/Ns are given, setting the value obtained for planar silicon to 1. The data in Fig. 2 cannot be directly compared with that of Fig. 1 as different scanner settings were applied. Representative data from one out of three or more experiments are shown. *Saturated microarray signals.

First, a wide variety of blocking reagents, based on proteins as well as polymers/detergents in different buffers (see Section 2), were evaluated. The results showed that a blocking solution based on 5% w/v fat-free milk powder in PBS was the preferred blocking agent in all cases except for FAST-slides (2% v/v Tween-20 in PBS) (data not shown). Similar results were obtained irrespective of the dye used (data not shown). Further, the composition of the washing solution and sample/secondary reagent buffer (denoted sample buffer) were also optimized. While the composition of the washing solution had less impact, 0.05% v/v Tween-20 in PBS was commonly used, the sample buffer had a major impact on reducing nonspecific background signals (data not shown). In general, 1% w/v fat-free milk powder, 1% v/v Tween-20 in PBS was found to be the optimal composition of the sample buffer.

Analysing Alexa-647-tagged serum, the results showed that the nonspecific background binding increased in the order of planar silicon < FAST-slides (new formula) < Maxisorb < Xenoslide N, FAST-slides (old formula) and protein binding slides (Fig. 2A). Targeting NHS-biotinylated serum showed a different ranking of the substrates, with the nonspecific background binding increasing in the order of Maxisorb < Nexterion slide H < protein binding slide < FAST-slide (new formula) < planar silicon < Xenoslide N < FAST-slide (old formula) (Fig. 2B). In general, lower nonspecific background signals were observed for NHS-biotin tagged serum than for Alexa-647 labeled serum (*cf.* Figs. 2A and B).

Focusing on NHS-biotinylated serum, we further examined the influence of the choice of solid support on our ability to target medium- and low-abundant analytes in crude serum (Fig. 2C). While the medium-abundant (nM range) serum analyte, C1q, could be detected on all surfaces, a significant difference was observed for the detection of pM to fM range targets, such as IL-6, IL-8, and IL-12. The performance of the substrates decreased in the order of Maxisorb and Nexterion slide H > FAST-slide (new formula) > protein binding slide > Xenoslide N > FAST-slide (old formula) > planar silicon. In accordance, the S/Ns observed for the substrates were found to decrease in the same order (Fig. 2C). Taken together, the results showed that antibody microarrays fabricated on black polymer Maxisorb slides (or Nexterion slide H) were the preferred choice when targeting rare protein targets in complete NHS-biotinylated proteomes.

3.4 Choice of solid support – Maxisorb versus Nexterion slide H

To further examine the performance of black polymer Maxisorb slide (Fig. 3A) and Nexterion slide H (Fig. 3B), we compared the dynamic span region for the substrates. To this end, the signal intensities observed for low- and high-abundant analytes targeted on a microarray scanned at different scanner settings were compared. Similar results were

obtained if serial dilutions of the samples were analyzed instead (data not shown). Representative results, illustrated by C3 and VEGF, are shown in Fig. 3. In comparison, lower scanner settings could be used on the Nexterion slide H than on the black polymer Maxisorb to detect the protein analytes at a higher sensitivity (*cf.* Figs. 3A and B). However, the signals were more rapidly saturated on Nexterion slide H than on black polymer Maxisorb slides, implying a more limited working dynamic range. As we aimed for a surface providing sensitive assays with as large dynamic span as possible, we selected black polymer Maxisorb slides as our preferred substrate for complex proteome analysis.

3.5 Degree of labeling

Next, we examined the influence of the degree of labeling on the detection of low-abundant analytes in complex proteomes. Human serum was NHS-biotinylated at a set of biotin/protein ratios, ranging from 2:1 to 450:1, and the content of IL-4 (Fig. 4A), IL-2 (Fig. 4B), IL-8 (Fig. 4C), and VEGF (Fig. 4D) were determined using recombinant antibody microarrays generated on black polymer Maxisorb slides.

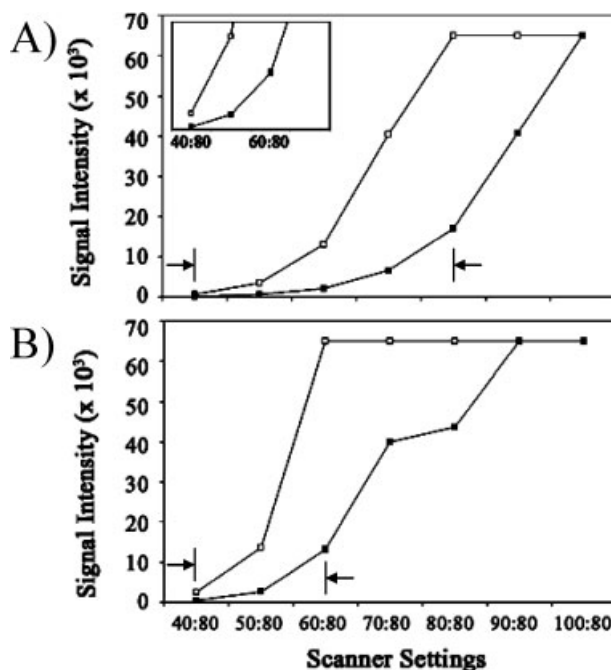


Figure 3. Comparison of the dynamic span region for black polymer Maxisorb slides and Nexterion slide H. The signal intensities observed for C3 (open squares) and VEGF (black squares) on (A) black polymer Maxisorb slides, (B) Nexterion slide H scanned at different scanner settings are shown. The signal intensities shown represent the mean value of eight replicates after subtracting local background and negative control. The potential working dynamic span region is marked by two arrows. In (A), a blow-up of the low signal intensity region is included. Representative data from one out of three experiments are shown.

The results showed that the signal intensities peaked at a molar ratio of dye/protein of 15:1 (IL-4), 45:1 (IL-2 and IL-8), or 450:1 (VEGF) (Fig. 4). In addition, IL-3 and IL-5 were found to peak at a labeling ratio of 45:1 (data not shown). Furthermore, similar curves were in all cases observed when looking at the S/Ns instead of the signal intensities (data not shown). Hence, the results indicated that 45:1 was a suitable labeling ratio in order to both optimize the detection (sensitivity) and to minimize the risk of over labeling the sample resulting in reduced sensitivity.

3.6 Specific and selective detection

Finally, we examined the specificity and selectivity of our recombinant antibody microarray set-up targeting rare protein analytes in complex proteomes (Fig. 5). NHS-biotinylated human serum was separately spiked with pure, labeled (100 pg/mL) or unlabeled (10 pg/mL) cytokines, including IL-1 α , IL-6, IL-8, IL-12, TNF- β , or VEGF and the signal intensities were compared to those of the nonspiked serum, respectively. In all cases, the results showed that the signal intensities increased or decreased in a specific and dose-dependent manner. No crossreactivity was observed since the signal intensity for a particular antibody only changed when the cognate antigen was spiked in (data not shown). A representative microarray image obtained for the nonspiked serum is shown in Fig. 5B, demonstrating the high quality (e.g., high spot morphology and low off-spot binding) of the arrays using the optimized array set-up. Hence, the results showed that we had achieved a highly selective and specific set-up for detection of low concentrated analytes in complex proteomes.

In conclusion, we have designed the first generation of a high performing recombinant antibody microarray technol-

ogy platform on black polymer Maxisorb slides for detection of low-abundant protein analytes in complete, *i.e.*, non-fractionated, NHS-biotinylated complex proteomes.

4 Discussion

Turning to the proteome, it has become evident that novel high-performing proteomic technologies will be required [2, 7, 9]. In particular, the need for high-throughput methodologies capable of targeting rare (pg/mL) protein analytes in a multiplexed, specific and sensitive manner in a complex setting (e.g., in crude human serum) is tremendous. Major efforts are currently under way by us [29–34, 36] (Steinhauer, C., Borrebaeck, C. A. K., Wingren, C., 2007, submitted) and others [20, 23–25, 40–44] to develop the antibody-based microarray technology into the high-throughput proteomic research tool needed [9, 17, 28]. We have focused our efforts on designing high-performing recombinant scFv antibody microarrays. To succeed with this challenge, we have taken on a comprehensive approach, and are addressing most of the key issues, including (i) content [31, 32, 36], (ii) solid support [30, 33], (iii) array format/fabrication [37, 38, 45], (iv) sample [29, 46], (v) analytical principle [33, 34, 47], and (vi) data processing [46]. In this study, we have addressed two of these issues, including choice of sample labeling-tag and solid support.

The labeling of complex proteomes is in itself a challenging task [9]. The problem could be bypassed by adopting a sandwich approach [48] or by interfacing a label-free analytical principle [34, 47, 49]. However, the former design is not compatible with scaling up the arrays [9, 17, 19], and the latter approach, although promising, will require more work before it could readily be combined with the multiplexed and

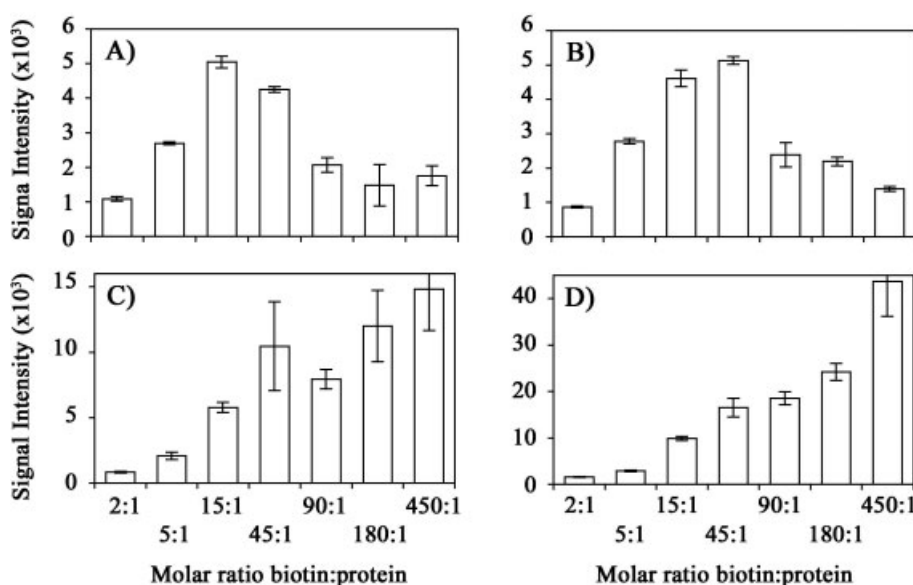


Figure 4. Influence of the degree of NHS-biotinylation on the detection of low-abundant protein analytes in complex proteomes. Human serum was NHS-biotinylated at different molar ratios of biotin to protein, ranging from 2:1, 5:1, 15:1, 45:1, 90:1, 180:1 to 450:1 and analyzed on recombinant antibody microarrays fabricated on black polymer Maxisorb slides. (A) Detection of IL-4, (B) detection of IL-2, (C) detection of IL-8, and (D) detection of VEGF. The array signal intensities shown represent the mean value of eight replicates after subtracting local background and negative control. Representative data from one out of three experiments are shown.

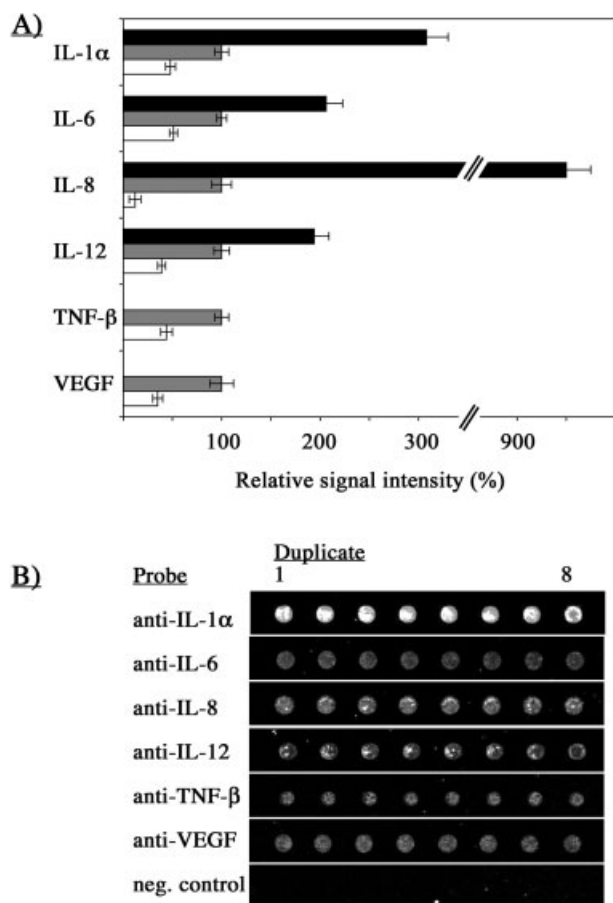


Figure 5. Specific and selective detection of low-abundant protein analytes in NHS-biotinylated crude human serum analyzed on black polymer Maxisorb slides. (A) Evaluation of the specificity by spiking the serum sample (gray bars) with pure labeled analyte (black bars) or pure unlabeled analyte (open bars). (B) Microarray images obtained for the nonspiked NHS-biotinylated human serum in (A). The array signal intensities shown represent the mean value of eight replicates after subtracting local background and negative control. Representative data from one out of three experiments are shown.

miniaturized format of microarrays. Still, we readily observed an LOD in the pM to sub-fM range targeting low-abundant analytes in directly labeled complex proteomes, e.g., crude human serum, (Table 1 and Fig. 2) clearly showing that we have developed a high-performing labeling protocol.

Adopting a label-based read-out mode, several microarray designs successfully rely on a dual color approach [27, 50, 51]. However, in agreement with recent observations [27, 50], we found that the signal intensities observed for the two dyes of each pair differed significantly (in average a 3–6 times difference was observed) (Fig. 1), clearly indicating on inherent differences in labeling efficiency or “transmitted signal intensities”. In fact, criss-cross labeling is commonly adopted in order to supersede this problem [27, 49]. Further developments of the dye reagents may also improve the

skewed performance (e.g., www.kreatech.com). However, in our case the signal intensities of the least performing dye of each pair was very low (Fig. 1), so even if adopting a criss-cross labeling approach, which would have doubled the number of arrays to be handled, it would more or less have been like running a one-color set-up. Consequently, we adopted a true one-color approach for complex proteome analysis on our recombinant antibody microarrays.

The precise choice of single dye was found to have a significant impact on the LOD, with NHS-biotin (and ULS-biotin) being one of the top candidates (Table 1 and Figs. 1 and 2). This observation could, at least to some extent be explained by differences in labeling efficiency, dye stickiness (as displayed by a lower nonspecific background binding), and/or our ability to remove unreacted dye. In agreement, recent microarray studies have also found biotin to be an excellent choice of sample labeling-tag [24, 26, 44]. Interestingly, NHS-biotin and ULS-biotin displayed similar performance (Fig. 1) although (i) they labeled different amino acids on the surface of the proteins, lysines vs. cysteines, histidines, and methionines, respectively (www.piercenet.com and www.kreatech.com), and (ii) that labeled samples, e.g., serum, were analyzed at different dilutions (nine times differences). Further work will be required to explore the differential use of either type of biotin when targeting complex proteomes, focusing on for example rare low molecular weight protein analytes (Fig. 1, www.kreatech.com). Apart from the choice of dye, the degree of label to protein was also found to be crucial in order to maintain immunoreactivity (Fig. 4). While other groups have used a ratio of label to protein of about 10:1 [24, 26, 44], we found 45:1 to be the optimal choice. To some extent, this difference in selected labeling ratio could be explained by the fact that they used a different set-up (e.g., solid support) and/or targeted mainly high-abundant (> μ M) protein analytes (e.g., CRP, Igs, etc.), while we addressed truly low-abundant (pg/mL) analytes (e.g., cytokines) on black polymer Maxisorb slides.

To date, a range of solid supports have been developed and to various degree been evaluated for protein microarray applications [20, 22, 30, 52]. In this study, we included a set of solid supports known to perform well [20, 30], and that displayed different surface structures (e.g., 1-D vs. 3-D), surface chemistries (e.g., glass vs. polymer vs. NC), and immobilization chemistries (adsorption vs. covalent chemistry vs. affinity binding) (see Section 2). The results showed that the choice of surface had a major impact on the performance of our recombinant antibody microarrays, as judged by the nonspecific background binding signals and LODs (cf. Table 1 and Figs. 1 and 2). We found black polymer Maxisorb slides (planar/proprietary/adsorption) and Nexterion slide H (3-D surface/hydrogel/covalent binding) to be the best performing slides. In more detail, adequate spot morphologies (e.g., Fig. 5B), high S/Ns and outstanding LODs (cf. Table 1 and Fig. 2) were observed. The superior behavior could be explained by a combination of high biocompatibility and an outstanding ability to completely block, or minimize, non-

specific background binding. In agreement, black polymer Maxisorb slides have been shown to perform well in recent studies [20]. Furthermore, our results showed that while Nexterion slide H may provide lower LODs than black polymer Maxisorb slides (Fig. 4), its working dynamic range was in this study more limited. Recent work has also implied that Nexterion slide H indeed displayed a more limited dynamic range working area (Dr. R. Dietrich, personal communication). In the long run, this may require each sample to be analyzed at several dilutions, resulting in logistic limitations for large-scale studies. Recently, we have found black polymer Maxisorb slides to perform well also when targeting medium-abundant protein analytes in crude samples [46]. As our goal was to perform sensitive protein expression profiling of complete proteome, *i.e.*, targeting analytes over a wide range of concentrations, running as few slides as possible, we selected black polymer Maxisorb slides as our preferred choice of surface.

The combination of sample labeling-tag and solid support pin-pointed in this study provided an outstanding sensitivity in the pM to sub-fM range (Table 1). To the best of our knowledge these are among the best LODs reported for (recombinant) antibody microarrays targeting complete directly labeled proteomes [21, 53–55]. In fact, these LODs are already within the suggested range of sensitivities (ng/mL–pg/mL) required in order to be able to perform sensitive proteome analysis and detect clinically relevant biomarkers [12, 43]. Even more importantly, it should be emphasized that these analysis were performed on complete, *i.e.*, non-fractionated, complex proteomes. In comparison, most, if not all, conventional proteomic approaches require the sample to be prefractionated prior to the analysis in order to reduce the sample complexity [12, 14, 56, 57]. Similarly, we also had to prefractionate the sample on our early antibody microarray designs [29]. However, all such prefractionation strategies suffer from low yield and impaired reproducibility [12, 14], making them less attractive. In this study, we have thus designed a platform capable of handling non-fractionated proteomes that will be of importance for antibody microarray-based proteome analysis. In contrast to already existing platforms targeting mainly high to medium abundant analytes in crude samples [25, 58], this platform was also capable of addressing low-abundant analytes.

While the affinity of the antibody probes used is an important parameter, we have not found a direct correlation between the affinity constant and the LOD for a particular antibody in practical microarray applications [33, 46]. The LOD is probably dependent on a combination of several additional factors, such as how (well) the analyte is labeled in a complex sample *etc.* Moreover, spiking experiments showed that our recombinant antibody microarray platform displayed a high selectivity and specificity (Fig. 5). Previously, the functionality and specificity of antibody microarrays have been argued [59, 60]. However, by using antibodies, microarray adapted by design, *i.e.*, molecules designed to display the desired properties in microarray applications [31, 36], on

an in parallel optimized set-up, *e.g.*, using highly biocompatible solid supports, the functionality and/or specificity of arrayed antibodies should no longer be a major issue.

Taken together, we have designed the first generation of a high-performing recombinant scFv antibody microarray technology platform for rapid, sensitive, and multiplexed profiling of low-abundant protein analytes in non-fractionated biotinylated complex proteomes. This will allow us to generate pattern of expression profiles of selected biomarkers that could have a major impact on the field of (onco)proteomics enhancing our capability of performing disease diagnostics and prognostics, screening, as well as patient stratification.

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5 References

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