Microarrays based on affinity-tagged single-chain Fv antibodies: Sensitive detection of analyte in complex proteomes

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Protein-based microarrays are among the novel class of rapidly emerging proteomic technologies that will allow us to efficiently perform global proteome analysis. However, the process of designing adequate protein microarrays is a major inherent problem. In this study, we have evaluated a protein microarray platform based on nonpurified affinity-tagged single-chain (sc) Fv antibody fragments to generate proof-of-principle and to demonstrate the specificity and sensitivity of the array design. To this end, we used our human recombinant scFv antibody library genetically constructed around one framework, the n-CoDeR library containing $2 \times 10^{10}$ clones, as a source for our probes. The probes were immobilized via engineered C-terminal affinity tags, his- or myc-tags, to either Ni²⁺-coated slides or anti-tag antibody coated substrates. The results showed that highly functional microarrays were generated and that nonpurified scFvs readily could be applied as probes. Specific and sensitive microarrays were obtained, providing a limit of detection in the pM to fM range, using fluorescence as the mode of detection. Further, the results showed that spotting the analyte on top of the arrayed probes, instead of incubating the array with large sample volumes (333 pL vs. 40 μL), could reduce the amount of analyte required 4000 times, from 1200 attomole to 300 zeptomole. Finally, we showed that a highly complex proteome, such as human sera containing several thousand different proteins, could be directly fluorescently labeled and successfully analyzed without compromising the specificity and sensitivity of the antibody microarrays. This is a prerequisite for the design of high-density antibody arrays applied in high-throughput proteomics.

Keywords: 
Affinity-tag / Antibody chips / Antibody microarrays / Sensitivity / Single-chain Fv

1 Introduction

Entering the postgenomic era, proteomics, the large-scale analysis of proteins, has become a key discipline for identifying, characterizing, and screening all proteins encoded by the genome. The human proteome is believed to be composed of $>30,000$ different proteins, distributed among approximately 200 different cell types. In analogy to the DNA
microarray technology, protein microarrays outline a unique possibility to develop a rapid global analysis of the entire proteome [1–5]. Protein microarrays will provide high-throughput means to perform comparative proteome analyses of, e.g. healthy and diseased cells [6–8]. This will allow scientists to identify disease-specific proteins, and to perform disease diagnostics and biomarker discovery that in the end will form a novel base for the development of future therapeutic principles.

In order to perform true global proteome analysis, high demands will be placed upon the choice of catcher proteins, or probes. To this end, molecular libraries providing numerous probes based on a single scaffold, i.e. with similar biophysical properties, will display significant advantages [9]. Antibody libraries [10, 11] are commonly identified as the preferred choice [1–3], since antibodies display a stunning specificity, distinguishing, for example, between single functional groups. The specificity of the probes is in fact a critical feature, since analytes must be specifically detected also in heterogeneous mixtures, such as crude cell extracts, containing >10 000 different irrelevant proteins [12]. To date, mainly low-density antibody microarrays have successfully been designed and developed [6–8, 13–15]. These arrays have provided proof-of-principle for the technology and experimentally indicated the significant potential of the approach.

However, proteome analyses are complicated by the fact that about 90% of proteins make up only 10% of the total protein mass [16]. In many cases, the proteins of interest will, thus, be present only with a few molecules per cell. In view of this, the sensitivity of antibody microarrays should be at least in the picogram range (attomole range). Theoretically, a detection limit of about 10 6 M can be proposed for antibody arrays [17]. To date, most published antibody microarray systems report a LOD in the nanogram range [15]. More specifically, LODs in the nM to pM range has been reported for set-ups detecting directly fluorescently labeled protein analytes [14, 18, 19]. We have recently demonstrated specific and sensitive detection of protein analyte in the 600 attomole range (sub-nM range), using MALDI-TOF MS as the mode of detection [13]. By adopting antibody sandwich assay set-ups combined with various signal amplification approaches, such as rolling-circle amplification, LODs in the pm to fm ranges have been reported for protein analytes [20–23]. Although sandwich assay set-ups may enhance the sensitivity (and specificity) of antibody microarrays, this design is not compatible with generating high-density arrays, since the task of generating adequate antibody pairs for numerous analytes will be overwhelming.

The issue of scaling up the arrays to a high-density format (>1000 probes) is in fact a key technology feature that needs to be fully addressed before global proteome analysis can be performed. The logistics behind designing high-density arrays will require nonpurified probes to be directly arrayed. To date, mainly prepurified probes have been applied. Arraying nonpurified probes may in fact be advantageous, since the presence of irrelevant (carrier) proteins have been found to increase the yield of dispensing minute volumes of probe [24]. To this end, the engineering of affinity-tagged probes and matching biocompatible surfaces may allow crude probe preparations to be purified, enriched and coupled in a one-step procedure directly on the chip. Substrates modified with Ni2+-ions [25, 26] or streptavidin [27, 28] are examples of surfaces that have been successfully applied to generate protein arrays through specific coupling chemistries. In addition, such optimized immobilization strategies may allow the probes to adopt a more favorable orientation, thereby generating highly functional arrays [29].

In this study, we have generated proof-of-principle for a protein microarray design based on nonpurified affinity-tagged single-chain (sc) Fv antibody fragments attached through specific affinity interactions as probes. We have used our human recombinant scFv antibody library genetically constructed around one framework, the n-CoDeR library containing 2 × 108 clones [10], as a source for our probes. The probes were immobilized via engineered C-terminal affinity tags, his- or myc-tags, to either Ni2+-coated slides or pre-arrayed intact monoclonal anti-tag antibodies. The results showed that specific and sensitive antibody microarrays were obtained, providing assay sensitivity in the pM to fm range, without using any signal amplification. In addition, highly complex proteomes could be analyzed without impairing the specificity and sensitivity of the set-ups.

2 Materials and methods

2.1 Proteins

Fourteen human recombinant scFv antibody fragments directed against eight human complement factors (CFs); C1q (clone C1q-4), C1s (clone C1s-8), C3 (clones C3-7 and C3-28), C4 (clone C4-3), C5 (clones C5-9 and C5-12), C1 esterase inhibitor (EI) (clone EI-12), factor B (FB) (clone FB-7) and properdine (FP) (clone Prop-3), and against four additional antigens; interleukin (II)-6 (clone IL-6-64), choleratoxin subunit B (CT) (clone CT-17), mucine-1 (clone SMUC-159) and FITC (clone FITC-8), were selected from the n-CoDeR library [10]. All scFvs carried a C-terminal myc-tag (EQKLI-SEEDL) followed by a his-tag. The affinities (Kd values) of the scFvs decreased in the order C1q-4 (2 × 10–10 M) > FITC-8 (0.9 × 10–9 M) > FB-7 (1 × 10–9 M) > Prop-3 (1 × 10–9 M) > CT-17 (3 × 10–9 M) > C4-3 (8 × 10–9 M) > SMUC-159 (2 × 10–8 M) > C5-9 (5 × 10–8 M) > EI-12 (7 × 10–8 M) > C3-21 (5 × 10–7 M) [10, 30, 31]. Purified human C1q, C3, C5, FB and FP were purchased from Quidel (San Diego, CA, USA). Purified CT and biotinylated FITC were obtained from Sigma (St. Louis, MO, USA). Synthetic peptides (60-mer) of mucine-1 [32] were biotinylated at their N-terminus. Normal human sera were obtained from healthy blood donors. In normal human sera, the concentration of CFs decreases in the order C3 (6500–8100 nM) > FB (2100–5500 nM) > C4 (1700–
2.2 Production and purification of scFv

All scFv antibodies were produced in *Escherichia coli*. Briefly, cell cultures were grown in 2 × (YT) 10 g bacloctryptone/2, 10 g yeast extract/2, 5 g NaCl/2, pH 7.0 medium containing 100 μg/mL ampicillin at 37°C until an OD₆₅₀ of 0.5 was obtained and then induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside, followed by overnight incubation at either 37°C (for scFv expressed in the supernatant) or 30°C (for scFv expressed in the periplasmic space). The expression supernatants were subsequently stored at 4°C until further use. The periplasmic fractions were either (i) dialyzed against PBS and stored at 4°C until further use or (ii) further purified by Ni-NTA chromatography (Qiagen). Bound molecules were eluted with 250 mM imidazole (in 50 mM sodium phosphate, 300 mM sodium chloride, pH 8.0), dialyzed against PBS and then stored at 4°C until further use. The activity and levels of expressed scFv were evaluated by means of 12% SDS-PAGE, Western blot, microBCA and dot-blot analyses. Briefly, samples were applied in serial dilutions. The SDS-PAGE gels were stained with CBB. The Western blots were processed according to standard protocols, using either anti-his or anti-c-myc mAb and horseradish peroxidase-conjugated rabbit anti-mouse Ig antibodies (DAKO, Glostrup, Denmark). The color reactions were developed with an ECL substrate kit (Amersham Biosciences, Solna, Sweden). The dot-blots were processed in either a similar manner or developed by adding Cy5-labeled antigen and subsequently scanned using a confocal fluorescence scanner (ScanArray Express, Perkin Elmer, Boston, MA, USA). The protein concentration was determined using a micro bicinchoninic acid Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

2.3 Cy5-labelling

CT and three pure CFs, C5, FB and FP, were labeled with Cy5 mono-reactive dye following the recommendations of the manufacturer (Amersham Biosciences). Briefly, the analytes were labeled in 0.1 m sodium carbonate, pH 9.3 at a ratio of mg protein to mg dye of 1 for 30 min at room temperature. Next, the mixtures were extensively dialyzed against PBS and subsequently stored at 4°C until further use. In order to retain the reactivity of C3 and C1q with their corresponding scFv antibodies, the Cy5-labelling conditions were modified. C3 was labeled in PBS at a ratio of mg protein to mg dye of 0.5 for 3 h at room temperature, where after the sample was dialyzed against PBS and stored at 4°C. C1q was labeled in 40% glycerol, PBS at a ratio of mg protein to mg dye of 0.5 for 7 h at 4°C. Finally, the mixture was dialyzed against 40% glycerol, PBS, and then stored at 4°C until further use. Human serum, diluted 3.5 times in PBS, was labeled at a ratio of mg protein to mg dye of 0.5 for 7 h at 4°C. Next, the mixture was extensively dialyzed against PBS and then the labeled sera was used immediately.

2.4 Production and evaluation of scFv microarrays

All scFv microarrays were generated using the non-contact printer Biochip Arrayer1 (Perkin Elmer), which deposits 333 pL sample/drop using piezo technology. Unless otherwise stated, the scFvs were coupled via one of their affinity tags, either (i) directly to Ni²⁺-chelate coated glass slides (Xenopore, Hawthorne, NJ, USA) (denoted Ni²⁺-slides), or (ii) to pre-arrayed monoclonal anti-tag antibodies (anti-his or anti-myc) deposited on derivatized aminosilane (SilanPrep) glass slides (Sigma) (denoted silane-slides) or on NC glass slides (FAST Slides; Schleicher and Schuell, Dassel, Germany) (denoted FAST-slides). In one experiment, the scFvs were directly adsorbed onto NC coated silica slides (denoted silica-slides) [13, 34]. In all arrays, eight replicates of each spot were generated to provide a sufficient number of replicates to ensure adequate statistics. The developed slides were scanned at 5 μm resolution using a nonconfocal fluorescence scanner (ScanArray Express microarray scanner; Perkin Elmer). The ScanArray Express software V2.0 (Perkin Elmer) was used to quantitate the intensity of each spot using the fixed circle method. Each data point presented represents the median value of eight replicates after subtracting local background and/or blank intensities. The detection limit was defined as the signal intensity of the negative control (nonspecific scFv) plus 2 SDs. A two-tailed t-test was used to determine whether the observed signals were significantly above the limit of detection (*p < 0.05*). Only data found to be above the limit of detection were presented. Three general array set-ups were designed depending on the mode of deposition (spotting or incubation) of the anti-tag antibody, the probe, and the analyte; (i) a non-multiplexing set-up (anti-tag antibody-spotting, probe-incubation, analyte-incubation), (ii) semimultiplexing set-up (spotting-spotting-incubation) and (iii) multiplexing set-up (spotting-spotting-spotting). Irrespective of the mode of deposition, the term probe was exclusively used for the content, i.e., the scFvs, and the term analyte was always defined as the molecule (sample) to be assayed.

2.4.1 Ni²⁺-slides

Ni-NTA purified scFv (1–8 femtomole; clones FITC-8, SMUC-159, CT-17, FB-7 and C1q-4) was arrayed by spotting 1 to 4 drops on top of each other (the spots were allowed to dry out in between). Four femtomole of a nonspecific scFv (clone FITC-8 or CT-17) was used as a negative control. Peri-
plasmic scFv preparations (clones FITC-8, CT-17, FB-7 and C1q-4) were arrayed by spotting 3 or 4 drops on top of each other (the spots were allowed to dry out in between). Similarly, a model periplasmic preparation, generated by spiking Ni-NTA purified scFv (clone FB-7, 400 μg/mL) 1:1 with 0.01 to 1.0% w/v fat-free milk powder in PBS (corresponding to 20 to 96 μg milk proteins per μg scFv), was arrayed. The arrays were blocked with 5% fat-free milk powder in PBS (blocking solution) for 1 h. All incubations were conducted in a humidity chamber at room temperature. Subsequently, the arrays were washed three times with 0.1% fat-free milk powder in a humidity chamber at room temperature. Next, the arrays were washed four times with washing solution. In the case of biotinylated antigen, serial dilutions (300 zeptomole to 60 attomole) on top of the nonpurified (periplasmic) preparations of the individual (or mixed) scFv periplasmic preparations (clones C1q-4, C1s-8, C3-7, C3-28, C4-3, C5-9, C5-12, EI-12, FB-7, Prop-3 and CT-17) for 1 h at room temperature, after which the slides were washed four times with washing solution. Finally, the slides were allowed to dry out and immediately scanned.

2.4.2 Silane- and FAST-slides

The monoclonal anti-tag antibodies (30–300 attomole), anti-his (5–40 pg) or anti-myc (30–50 pg), were arrayed by spotting single drops. Subsequently, two approaches for depositing the probes were applied. In the non-multiplexing set-up, the slides were blocked and washed as described above (see Section 2.4.1). Next, each array was incubated with 50 μL of individual (or mixed) scFv periplasmic preparations (clones C1q-4, C1s-8, C3-7, C3-28, C4-3, C5-9, C5-12, EI-12, FB-7, Prop-3 and CT-17) for 1 h at room temperature, after which the arrays were washed four times with washing solution. Nonspecific scFv in the format of 0.5 to 5 femtomole Ni-NTA purified scFv (clones FITC-8 and IL-6-64) or periplasmic fractions (clones CT-17, FITC-8, IL-6-64) were used as negative controls. No signals were obtained for pre-arrayed anti-tag antibodies incubated with pure Cy5-labelled antigens. In the semimultiplexing set-up, the probes (Ni-NTA purified (0.5 to 5 femtomole), periplasmic fractions or expression supernatants) were arrayed on top of the anti-tag antibodies by spotting four drops (two drops were applied and allowed to dry out before the next two were applied). Nonspecific scFv in the format of 0.5 to 5 femtomole Ni-NTA purified scFv (clone CT-17, IL-6-64) and periplasmic fraction (clones CT-17, IL-6-64, as well as vector without any scFv insert) were used as negative controls. PBS spotted on top of pre-arrayed anti-tag antibodies were used as the blank. The arrays were then blocked and washed as described above (see Section 2.4.1). Next, the arrays from either set-up were incubated with 50 μL 4.3 pm to 80 nm Cy5-labeled analyte in PBS (or spiked into 5% fat-free milk powder in PBS). The array was washed four times with washing solution, allowed to dry out and then the slides were immediately scanned and any bound analyte was detected.

2.4.3 Silica-slides

Ni-NTA purified scFv (2 femtomole; clone CF-17) was arrayed as single spots. Identical amounts of Ni-NTA purified nonspecific scFv (clone FITC-8) were used as negative controls. The array was blocked and washed as described in Section 2.4.1, before Cy-5 labeled antigen (CT) was spotted in serial dilutions (300 zeptomole to 60 attomole) on top of the arrayed probes in a true multiplexing approach. Subsequently, the slide was washed four times in washing solution, allowed to dry out and then the slides were immediately scanned and any bound analyte detected.

3 Results

Antibody microarrays can be designed in a number of different formats depending on the application. To evaluate and derive optimal efficacy, we have designed antibody microarrays based on purified as well as nonpurified human recombinant affinity-tagged scFv antibody fragments. The probes were immobilized through specific affinity interactions to various substrates. The array designs were evaluated to generate proof-of-principle and to demonstrate the specificity and sensitivity of each individual set-up.

3.1 Limit of detection

3.1.1 Ni2+-coated substrates

The LOD was first determined using five pure affinity-tagged scFvs against analytes of various sizes, ranging from small hapten to large proteins, as model probes (Fig. 1). The LODs were found to vary between 43 to 2000 pm, corresponding to 175 to 9200 attomole analyte/spot, using pure labeled analytes (Fig. 1A). Representative microarrays (Fig. 1B) and matching signal intensity plots (Fig. 1C) are shown for the anti-C1q-C1q system. Adequate spot morphologies, low background and low nonspecific binding were observed (Fig. 1B). No direct correlations between either the size of the analyte or antibody affinity (see Section 2.1) and LOD was observed. Still, the scFv with the highest affinity (clone C1q-4) that was directed against the largest antigen gave the lowest LOD. Taken together, the results showed that sensitive microarrays based on pure affinity-tagged scFv could readily be generated on Ni2+-coated slides.

Next, the nonpurified (periplasmic) preparations of the same probes, except for SMUC-159, were used to generate a similar set of microarrays. No signals were obtained for any of the arrays after incubation with 20–80 nm analyte (data not shown). The concentration of scFv in the periplasmic preparations was estimated to be about 50–100 μg/mL as determined by indirect dot-blot analyses, indicating that sufficient amount of probe (< 5 femtomole) should have been deposited to generate a detectable signal. In order to investi-
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3.1.2 Anti-tag antibody coated substrates

To design a format where nonpurified probes could be used, we applied anti-tag antibody coated substrates. To this end, intact monoclonal anti-myc or anti-his IgG antibodies were arrayed on either silane-slides or FAST-slides. Nonpurified (periplasmic) probe preparations were deposited through incubation on these pre-arrayed anti-tag antibodies, and the LODs were determined using pure, Cy5-labeled protein analytes (Fig. 2 and 3). The results showed that highly functional microarrays were generated and that nonpurified scFvs could readily be applied as probes (Figs. 2 and 3). In the case of silane-slides, LODs in the range of 43 pm to 110 nm, cor-

Figure 1. LOD for antibody microarrays based on pure affinity-tagged scFv antibodies on Ni²⁺-slides. Pure labeled analytes were applied. (A) LOD for probes directed against a hapten, peptide or protein analyte. (B) Cy5-scanned images of microarrays for the anti-C1q-C1q system. (C) Signal intensities for the Cy5-scanned microarrays of the anti-C1q-C1q system. The median values, based on eight replicates, are shown for each probe concentration (8 femtomole, white; 4 femtomole, grey; 2 femtomole, striped) after subtracting local background and any signal from the negative control (nonspecific scFv) plus two SDs (i.e. only signals defined as above LOD are shown).

Figure 2. LOD for antibody microarrays based on nonpurified (periplasmic preparations) affinity-tagged scFv antibodies attached via pre-arrayed monoclonal anti-tag antibodies (anti-myc or anti-his) on silane-slides. Pure Cy5-labeled analytes were applied. The probes were deposited through incubation on pre-arrayed anti-tag antibodies in a nonmultiplexing approach. (A) LOD for probes directed against various protein analytes. (B) Cy5-scanned images of microarrays for the anti-C1q-C1q system on anti-myc coated silane-slides. (C) Signal intensities for the Cy5-scanned microarrays of the anti-C1q-C1q system on anti-myc coated silane-slides. The median values, based on eight replicates, are shown for each probe concentration (330 attomole, white; 270 attomole, grey; 200 attomole, striped) after subtracting local background and any signal from the negative control (nonspecific scFv) plus two SDs (i.e. only signals defined as above LOD are shown).
Figure 3. LOD for antibody microarrays based on nonpurified (periplasmic preparations) affinity-tagged scFv antibodies attached via pre-arrayed monoclonal anti-tag antibodies (anti-myc or anti-his) on FAST-slides. Pure Cy5-labeled antigens were applied. The probes were deposited through incubation on pre-arrayed anti-tag antibodies in a nonmultiplexing approach. (A) LOD for probes directed against various protein analytes. (B) Cy5-scanned images of microarrays for the anti-C1q-C1q system on anti-his coated FAST-slides. (C) Signal intensities for the Cy5-scanned microarrays of the anti-C1q-C1q system on anti-his coated FAST-slides. The median values, based on eight replicates, are shown for each probe concentration (270 femtomole, white; 135 attomole, grey) after subtracting local background and any signal from the negative control (nonspecific scFv) plus two SDs (i.e. only signals defined as above LOD are shown).}

responding to 170 to 650 attomole analyte/spot, were obtained using anti-myc as the anti-tag antibody (Fig. 2A). Higher LODs, in the range of 430 fM to 90 nM, corresponding to 2.7 attomole to 570 femtomole analyte/spot, were obtained using anti-his as the anti-tag antibody. Compared with the Ni\textsuperscript{2+}-slides, equal (clone C1q-4) or better (FB-7) LODs were obtained on anti-tag coated silane-slides (Figs. 1 and 2). Representative microarrays, illustrated by those of anti-C1q on anti-myc coated slides are shown in Fig. 2B and C. The arrays displayed excellent spot morphologies, low background, and low nonspecific binding (Fig. 2B). No direct correlation between either size of the analyte or antibody affinity (see Section 2.1) and LOD was observed. Noteworthy, the assay sensitivity was improved 10 to 200 times when two probes (clones C5-9 and C5-12) directed against the same analyte (C5) were arrayed together in the same spot instead of separately (Fig. 2A). Thus, the results showed that cooperative binding effects might be utilized to further improve LOD.

Next, the LODs were determined on anti-tag antibody coated FAST-slides (Fig. 3). Based on the results obtained for the anti-C1q-C1q system (1000 times better; Fig. 3A), anti-his was found to be the preferred choice of anti-tag antibody. Using anti-his as the anti-tag antibody, LODs in the range of 430 fM to 90 nM, corresponding to 2.7 attomole to 570 femtomole analyte/spot, were obtained (Fig. 3A). Representative microarrays, illustrated by those of anti-C1q on anti-his coated slides, are shown in Fig. 3B and C. The arrays displayed excellent spot morphologies, low background and low nonspecific binding (Fig. 3B). As before (Figs. 1, 2 and 3), no direct correlation between either size of the analyte or antibody affinity (see Section 2.1) and LOD could be observed. Further, the assay sensitivity could be improved 40 times by depositing the two anti-C5 clones together instead of separately, further indicating the importance of cooperative binding effects on LOD (Figs. 2 and 3).

Noteworthy, the LODs were up to 1000 times lower on the FAST-slides than on the silane-slides (Figs. 2 and 3). Still, the scFvs were ranked in roughly the same order with respect to LOD. Interestingly, the anti-myc antibodies appeared to be the best choice of precatcher antibody on silane-slides (Fig. 2), while the anti-his antibody provided the most sensitive set-up on FAST-slides (Fig. 3).

### 3.2 Effect of probe format and deposition

To study the effect of probe format and deposition on signal intensities, different probe formats, Ni-NTA purified scFv, periplasmic scFv preparation, expression scFv supernatant were applied (Fig. 4). The probes were deposited through direct spotting (all formats, pL scale, semimultiplexing approach) or incubation (only periplasmic format, µL scale, nonmultiplexing approach) on pre-arrayed anti-tag antibodies on silane slides (Fig. 4A) and/or FAST-slides (Fig. 4B). The results showed that all three probe formats and both probe deposition methods could be used equally well to generate functional arrays (Fig. 4A and B). The actual signal intensities observed varied, but these variations correlated well with the concentration/amount of probe applied. Next, LOD was determined for microarrays based on nonpurified probes (periplasmic preparations) deposited through direct spotting on top of pre-arrayed anti-his antibodies on silane slides (Fig. 4A) and/or FAST-slides (Fig. 4B). The results showed that all three probe formats and both probe deposition methods could be used equally well to generate functional arrays (Fig. 4A and B). The actual signal intensities observed varied, but these variations correlated well with the concentration/amount of probe applied. Next, LOD was determined for microarrays based on nonpurified probes (periplasmic preparations) deposited through direct spotting on top of pre-arrayed anti-his antibodies on FAST-slides in a semimultiplexing approach (Fig. 4C). Anti-C1q-C1q was used as the model system. The results showed that an LOD of 2.1 pm, only 4.9 times higher
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Figure 4. Effects of probe format (degree of purity) and deposition on signal intensities for antibody microarrays based on affinity-tagged scFv antibodies attached via pre-arrayed monoclonal anti-tag antibodies. Three different probe formats, Ni-NTA purified scFv > periplasmic scFv preparation > expression scFv supernatant (ranked according to probe concentration), were used. Pure Cy5-labeled antigens were applied. (A) Signal intensities for different probe formats (pure, white; periplasmic preparation, grey and striped; expression supernatant, black) deposited through direct spotting (white, striped, black; semimultiplexing approach) or incubation (grey; nonmultiplexing approach) on pre-arrayed anti-tag antibodies on silane-slides. Anti-C1q-C1q was used as model system. (B) Signal intensities for pure (white) or periplasmic (grey) probe preparations deposited through direct spotting on top of pre-arrayed anti-his antibodies on FAST-slides in a semimultiplexing approach. Anti-CT-CT and anti-C1q-C1q were used as the model systems. (C) Signal intensities for periplasmic probe preparation deposited through direct spotting on top of pre-arrayed anti-his antibodies on FAST-slides in a semimultiplexing approach. Anti-C1q-C1q was used as the model system, and serial dilutions of antigen were applied. In all cases, the median signal intensities, based on eight replicates, are shown after subtracting local background and any signal from the negative control (nonspecific scFv) plus two SDs (i.e. only signals defined as above LOD are shown).

Figure 5. Effect of analyte deposition on LOD for antibody microarrays based on pure affinity-tagged scFv antibodies arrayed onto silica-slides. Pure Cy5-labeled antigen was deposited through direct spotting on top of the arrayed probes in a true multiplexing approach. Anti-CT-CT was used as the model system. The median signal intensities, based on eight replicates, are shown after subtracting local background and any signal from the negative control (nonspecific scFv) plus two SDs (i.e. only signals defined as above LOD are plotted).

3.3 Effect of analyte deposition

LOD was determined when the analyte was deposited through direct spotting on top of arrayed probes in a true multiplexing approach (Fig. 5). Anti-CT–CT on silica-slides was used as the model system. The results showed that an assay sensitivity corresponding to only 300 zeptomole (sub-nM) analyte was obtained. Although the LOD with respect to analyte concentration remained about the same (0.9 nm vs 0.5 nm), the amount of analyte required decreased significantly (300 zeptomole vs 1200 attomole) (Figs. 1 and 5). Thus, the results showed that adopting a multiplexing approach in which the analyte was spotted on top of the arrayed probes significantly reduced the amount of analyte required, an important feature when working with limited sample volumes.

3.4 Effect of analyte format

Identical signal intensities were obtained whether pure analyte or analyte spiked into human sera were analyzed on microarrays based on pure probes on Ni²⁺-coated slides (Fig. 6). Similar results were obtained irrespective of the size of the analyte, ranging from a small hapten to a large protein. Using C1q as the model protein, different analyte formats, pure or spiked into 5% fat-free milk powder, were analyzed on microarrays based on nonpurified probes (periplasmic preparations) immobilized via anti-his on silane-slides.
3.5 Detection of analytes in complex mixtures

Finally, human sera was directly Cy5-labeled and analyzed for content of various CFs using microarrays based on nonpurified probes attached via pre-arrayed anti-myc antibodies on silane-slides (Fig. 7). The results showed that seven of eight CFs targeted could be detected. Except for C3, the signal intensities obtained correlated with the anticipated

(Fig. 6B and C). Compared to analyzing pure analyte, the results showed that similar or slightly higher signal intensities were obtained for non-pure analyte. Identical analyses performed on anti-myc coated silane-slides showed that similar signal intensities were obtained irrespective of the analyte format (data not shown). Taken together, the results clearly showed that complex samples readily could be analyzed without compromising the specificity and sensitivity of the set-ups, an absolute prerequisite for working with complex proteomes.

![Figure 6](image_url)

**Figure 6.** Effect of analyte format (degree of purity) on signal intensities for antibody microarrays based on affinity-tagged scFv antibodies. (A) Signal intensities for three analytes, pure (white) or spiked into human sera (grey), on microarrays based on pure scFvs arrayed onto Ni\textsuperscript{2+}-slides. (B) Cy5-scanned images of microarrays for the anti-C1q-C1q system. Nonpurified probes (periplasmic preparations) were deposited through incubation on pre-arrayed anti-myc antibodies on silane-slides in a non-multiplexing approach. Cy5-labeled antigen, pure or spiked into 5% fat-free milk, was used. (C) Signal intensities for the Cy5-scanned microarrays for the anti-C1q-C1q system analyzed in (B). Signal intensities for pure (white) and non-pure (grey) analyte obtained at two probe concentrations (270 attomole and 135 attomole) are shown. In all cases, the median signal intensities, based on eight replicates, are shown after subtracting local background and any signal from the negative control (non-specific scFv) plus two SDs (i.e. only signals defined as above LOD are shown).

![Figure 7](image_url)

**Figure 7.** Detection of CFs in Cy5-labeled human sera using microarrays based on nonpurified (periplasmic preparations) affinity-tagged scFv antibodies against various complement proteins on silane-slides. The probes were deposited through direct spotting on top of pre-arrayed anti-myc antibodies in a semi-multiplexing approach. (A) Cy5 scanned image of the microarray. Blank, PBS; control 1, nonspecific scFv (periplasmic preparation) or blank periplasmic preparation (i.e. vector without scFv insert); control 2, nonspecific scFv (pure; no detectable signal intensity). (B) Signal intensities for the Cy5-scanned microarray. The CFs are plotted in order of decreasing serum concentration, from C3 (highest) to FP (lowest). Median values, based on eight replicates, are shown after subtracting local background and any signal from the blank (signal intensity of 80) and control 1 plus two SDs (signal intensity of 950; i.e. only signals defined as above LOD are plotted).
Protein Arrays

It should be noted that the arrayed anti-tag antibodies retained a high functionality although they dried out within a few seconds and were kept dry until (minutes to hours) the nonpurified probes were deposited. In contrast, proteins tend commonly to denature when arrayed onto a solid support and stored in a dehydrated state [2]. For example, Haab et al. [14] found that only 20% of the arrayed intact antibodies displayed an adequate activity. In this context, it should also be noted that our designed scFv probes displayed excellent on-chip stability, and arrayed dehydrated probes retained ≥ 80% of their activity for more than 200 days [9, Steinhauer et al., unpublished observations].

Using the anti-tag antibody coated substrates and nonpurified scFv as probes, set-ups providing a LOD in the pm to fs range could readily be obtained using fluorescence as the mode of detection with no further signal amplification (Figs. 2 and 3). To our knowledge, these are among the best LODs reported for such set-ups. In fact, these values are comparable to those LODs obtained for antibody microarrays where various signal amplification techniques have been applied [20–23, 35]. In our current set-up, the analytes were directly fluorescently labeled, an approach that may reduce the LOD depending on how efficiently the analyte (sample) can be labeled. In comparison, we have observed LODs in the attomole-range (sub-mM) (MALDI-TOF MS) [13], fs range (SELDI-TOF-MS) (Wingren et al., unpublished observations) and pm range (quartz crystal microbalance) (Larsson et al., unpublished observations) using label-free detection techniques. It has been proposed that the sensitivity of antibody microarrays should be at least in the picogram range (attomole range) to perform well in proteome analysis [15]. Here, we showed that the amount of analyte required was in the femtomole to zeptomole range (Figs. 2, 3 and 5). Thus, we have designed a microarray platform based on affinity tagged scFv providing sensitivity already within the suggested range, without using any specific signal amplification.

In this context, it may be of interest to compare these LODs with the best performing protein chips in general. Pawletz and coworkers [36] have observed LODs in the sub-zeptomole range using a RP protein microarray set-up, in which the analyte (prostatic specific antigen) was spotted and then subsequently detected using antibodies and signal amplification steps. Using an aptamer (short single stranded nucleic acid probes) based assay, IgE could be detected at a level of 37 zeptomole [37]. In a similar approach, but using arrayed allergens as probes, allergen-specific IgE could be detected at sub-zeptomole levels [38]. A direct titration of spotted Cy5 labeled IgG, using the planar waveguide technology, indicated a LOD of 2 pm corresponding to 0.8 zeptomole (500 protein molecules) [21]. Hence, the antibody-based microarrays described in the present study compares well with protein arrays, which normally are more sensitive due to less perturbation of the binding probes, pointing towards a molecular design of our scFv probes [10] well suited for the array format.

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Moreover, this study pointed to two separate ways by which LOD could be further improved. Firstly, by spotting at least two probes together in the same spot instead of separately, cooperative binding effects could be utilized to improve the LOD up to 200 times (Figs. 2 and 3). Cooperative binding effects is a well-known biochemical phenomenon, previously observed also for antibodies [e.g., 39], by which mixing two antibodies yields enhanced avidity for antigen, that may prove useful for future microarray set-ups. Secondly, by adopting a true multiplexing approach in which the analyte was spotted on top of the array instead of incubating the array with large sample volumes (333 µL vs 40 µL), the amount of analyte required could be reduced 4000 times, from 1200 attomole to 300 zeptomole (Figs. 1 and 5) [13]. Further experiments are required to evaluate and subsequently optimize the dynamic range for this and similar multiplexing approaches. The LODs observed were dependent on the choice of anti-tag antibody and the choice of substrate (Figs. 2 and 3). In the latter case, the LODs were up to 1000 times lower on FAST-slides than on silane-slides (Figs. 2 and 3). These observations reflected differences in (i) background, (ii) biocompatibility of the substrates and (iii) how well suited the commercially available anti-tag antibodies were to act as probes. In the latter case, it may be of interest to note that only 5% of over 100 commercially available antibodies recently tested were found to be suitable for microarray-based analyses (i.e., reagents not a priori selected and validated for chip applications) [2].

The LODs were not apparently affected by the sample complexity, as illustrated by the fact that similar fluorescence read-outs were obtained whether pure analyte or analyte spiked into complex sample, such as human sera, were analyzed (Fig. 6). However, the LODs may be reduced due to the fact that complex samples are difficult to label, especially if the target analyte(s) is present in low amounts. Still, we showed that CFs could be detected in directly Cy5-labeled human sera. In addition, we have recently shown that a set of cytokines could be detected after Cy5- and Cy3-labeling of crude cell extracts using antibody-based microarrays [12]. Moreover, recent results show that the sensitivity could be considerably enhanced by eliminating >95% of the irrelevant proteins in a one-step fractionation procedure prior to the sample labeling (Ingvarsson et al., unpublished observations).

When analyzing complex samples, the specificity of the probes will be a key issue. Recently, several studies have, however, raised doubts about the usefulness of antibodies in microarray formats because of concerns as to whether antibodies actually are sufficiently specific [2, 35, 40–42]. One explanation for this view is, as pointed out above [2], that in many cases commercially available antibodies have been directly applied without being tested and selected for their suitability to act as probes. We have recently shown that scFv probes from our n-CoDeR library provided a specific assay even when the arrays were incubated with crude extracts of human dendritic cells containing >10,000 different fluorescently labeled proteins [12]. In this paper, we also showed that human sera could be directly Cy5-labeled and analyzed for content of various CFs (Fig. 7). In this context, it should be pointed out that the pre-arrayed anti-tag antibodies displayed no or only low nonspecific binding (blank; Fig. 7). It was even more interesting to note that pure nonspecific scFv (control 2) displayed no nonspecific binding, while a low, but significant signal, was observed when the corresponding periplasmic preparation was used as control (control 1), indicating that protein(s) other than the scFv antibodies were responsible for the nonspecific binding observed (Fig. 7). Thus, the results showed that complex samples could readily be analyzed without compromising the specificity and sensitivity of the set-ups, supporting that recombinant scFv antibody fragments are well suited for the array format.

5 Concluding remarks

In conclusion, we have shown that specific and sensitive antibody microarrays could be generated using unpurified affinity-tagged scFv antibodies immobilized through specific affinity interactions. To enable such specific immobilization, we generated biocompatible anti-tag antibody coated substrates. By adopting a semimultiplexing approach using these substrates, dense arrays based on unpurified probes could readily be fabricated. The described logistically optimized approaches are necessary for utilizing high-density antibody microarrays in proteomics. The set-up also allowed us to specifically detect complement factors after direct labeling of the human serum proteome, demonstrating that most prerequisites are in place for high-throughput proteomics.

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6 References