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Affibody protein capture microarrays: Synthesis and evaluation of random and directed immobilization of affibody molecules

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Abstract

Affibody molecules, 58-amino acid three-helix bundle proteins directed to different targets by combinatorial engineering of staphylococcal protein A, were used as capture ligands on protein microarrays. An evaluation of slide types and immobilization strategies was performed to find suitable conditions for microarray production. Two affibody molecules, Z_{Taq} and Z_{IgA}, binding *Taq* DNA polymerase and human IgA, respectively, were synthesized by solid phase peptide synthesis using an orthogonal protection scheme, allowing incorporation of selective immobilization handles. The resulting affibody variants were used for random surface immobilization (through amino groups) or oriented surface immobilization (through cysteine or biotin coupled to the side chain of Lys^{58}). Evaluation of the immobilization techniques was carried out using both a real-time surface plasmon resonance biosensor system and a microarray system using fluorescent detection of Cy3-labeled target protein. The results from the biosensor analyses showed that directed immobilization strategies significantly improved the specific binding activity of affibody molecules. However, in the microarray system, random immobilization onto carboxymethyl dextran slides and oriented immobilization onto thiol dextran slides resulted in equally good signal intensities, whereas biotin-mediated immobilization onto streptavidin-coated slides produced slides with lower signal intensities and higher background staining. For the best slides, the limit of detection was 3 pM for IgA and 30 pM for *Taq* DNA polymerase.

Keywords: Affibody; Protein microarray; Immobilization; Biosensor

After the sequencing of the human genome, investigation of the corresponding proteome has become one of the major challenges in biochemical research. To be able to efficiently address protein function, expression, and localization on a proteome-wide scale, much effort has been directed toward the development of miniaturized and parallel assays. An important addition to this field is the protein capture microarray [1], which is analogous to the DNA microarray used for large-scale gene expression analysis and can be used for protein expression studies. Although still a young technology, protein capture microarrays have already been used successfully for the identification of differentially expressed biomarker proteins [2,3], and the technology holds great promise for the future.

To date, most protein microarrays have been constructed using antibodies as the specific capture agent [4], and microarray slides prepared with focused sets of antibodies are commercially available. However, the generation of specific antibodies against all human proteins required for global protein analysis is an enormous task [5], and selection of antibody fragments from recombinant libraries using in vitro methods such as phage display [6] has been suggested to be superior in terms of speed and efficiency. Another option is to use

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alternative capture agents, such as aptamers selected from nucleic acid libraries [7] and affinity proteins selected from libraries based on nonimmunoglobulin protein scaffolds [8], which could be more suitable for use in a microarray context.

One such type of affinity proteins selected from a combinatorial protein library are the affibody molecules, which are based on the 58-amino acid three-helix bundle protein scaffold of the Z domain, derived from staphylococcal protein A. Highly specific binders to a wide range of protein targets have been selected by phage display of libraries of affibody variants generated by randomization of 13 surface-exposed residues in helices 1 and 2 of the Z domain [9,10]. In contrast to antibodies, affibody molecules are small, lack disulfide bridges, and can be expressed in bacteria in high yields. Affibody molecules have been shown to function well in a variety of biotechnological applications based on molecular recognition [11–13] and have proved to be very stable in harsh conditions such as high pH [14]. Furthermore, because of the small size and ease of folding, an affibody can be synthesized by solid phase peptide synthesis [15], allowing straightforward introduction of unnatural amino acids as well as different reporter groups such as fluorophores and affinity handles for specific surface immobilization. Taken together, the properties of the affibody molecules suggest that they could be an attractive alternative to antibodies for use as capture agents in protein microarrays.

For the production of protein microarrays, an important issue is the strategy used for attachment of the protein on the chip surface. Immobilization of the protein by nonspecific adsorption is often associated with problems such as high background signal and loss of protein during stringent washes; therefore, specific attachment through covalent coupling or affinity interaction has been considered to be a better strategy [16]. It has been shown for antibodies that the orientation of the immobilized proteins affects their activities and that whole antibodies and Fab fragments specifically oriented with their binding sites away from the surface are more active and can be more densely packed than their randomly oriented counterparts [17]. In that work and in other studies, specific orientation of antibodies was achieved by noncovalent binding to the antibody-specific protein A or G or by covalent coupling to the carbohydrate moiety located in the Fc region or to free thiol groups liberated by mild reduction of interchain disulfide bonds [17,18]. For binding proteins that can be produced by chemical synthesis, a variety of other selective methods are available for surface immobilization. Different functional groups and affinity handles can easily be introduced in defined positions of the protein, allowing site-specific oriented coupling to the microarray surface.

In the current study, affibody molecules immobilized randomly or in a directed fashion were evaluated as capture agents on protein microarrays. Chemical synthesis was used for the preparation of affibody molecules modified with a thiol group or a biotin moiety, attached either directly to the side chain of a native C-terminal lysine residue or through an α-aminohexanoic acid (Ahx)[1] spacer, to increase the distance between the immobilized binding protein and the microarray surface (Fig. 1). The functions of the immobilized proteins have been evaluated in two different chip-based formats using surface plasmon resonance (SPR) and fluorescence for detection of bound target protein.

Materials and methods

Peptide synthesis reagents

Boc-Val-OH, Fmoc-Lys(Mtt)-OH, Fmoc-Ahx-OH, *O*-(1H-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), and 1-hydroxybenzotriazole (HOBt) were obtained from Calbiochem–Novabiochem (Läufelfingen, Switzerland). Trifluoroacetic acid (TFA), triisopropylsilane (TIS), and *tert*-butylmethyl ether were obtained from Merck (Darmstadt, Germany). D-Biotin (99%), *N*,*N*-diisopropylethylamine (DIEA), and piperidine were obtained from Sigma–Aldrich Chemie (Steinheim, Germany). All other peptide synthesis reagents, including Fmoc amide resin and standard side chain-protected amino acids, were obtained from Applied Biosystems (Warrington, UK).

Solid phase synthesis of affibody molecules

The affibody molecules Z_{Taq} ($Z_{Taq\ S1\text{-}1}$ in [19]), binding *Taq* DNA polymerase, and Z_{IgA} (Z_{IgA1} in [12]), binding human IgA, were synthesized by standard Fmoc chemistry [20] as described previously [15]. In brief, the proteins were synthesized with an Asp^2Glu substitution on an Fmoc amide resin (loading: 0.67 mmol g^{-1}) using an ABI 433A Peptide Synthesizer (Applied Biosystems, Foster City, CA, USA). All amino acids were standard Fmoc amino acid derivatives except Boc-Val-OH, used in position 1, and Fmoc-Lys(Mtt)-OH, used in position 58.

[1] *Abbreviations used:* Ahx, α-aminohexanoic acid; SPR, surface plasmon resonance; HBTU, *O*-(1H-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; TIS, triisopropylsilane; DIEA, *N*,*N*-diisopropylethylamine; DCM, dichloromethane; EDT, ethanedithiol; PBS, phosphate-buffered saline; RU, response units; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; NHS, *N*-hydroxysuccinimide; PDEA, 2-(2-pyridinyldithio)ethaneamine; CMD, carboxymethyl dextran; TD, thiol dextran; MES, 2-morpholinoethanesulfonate.

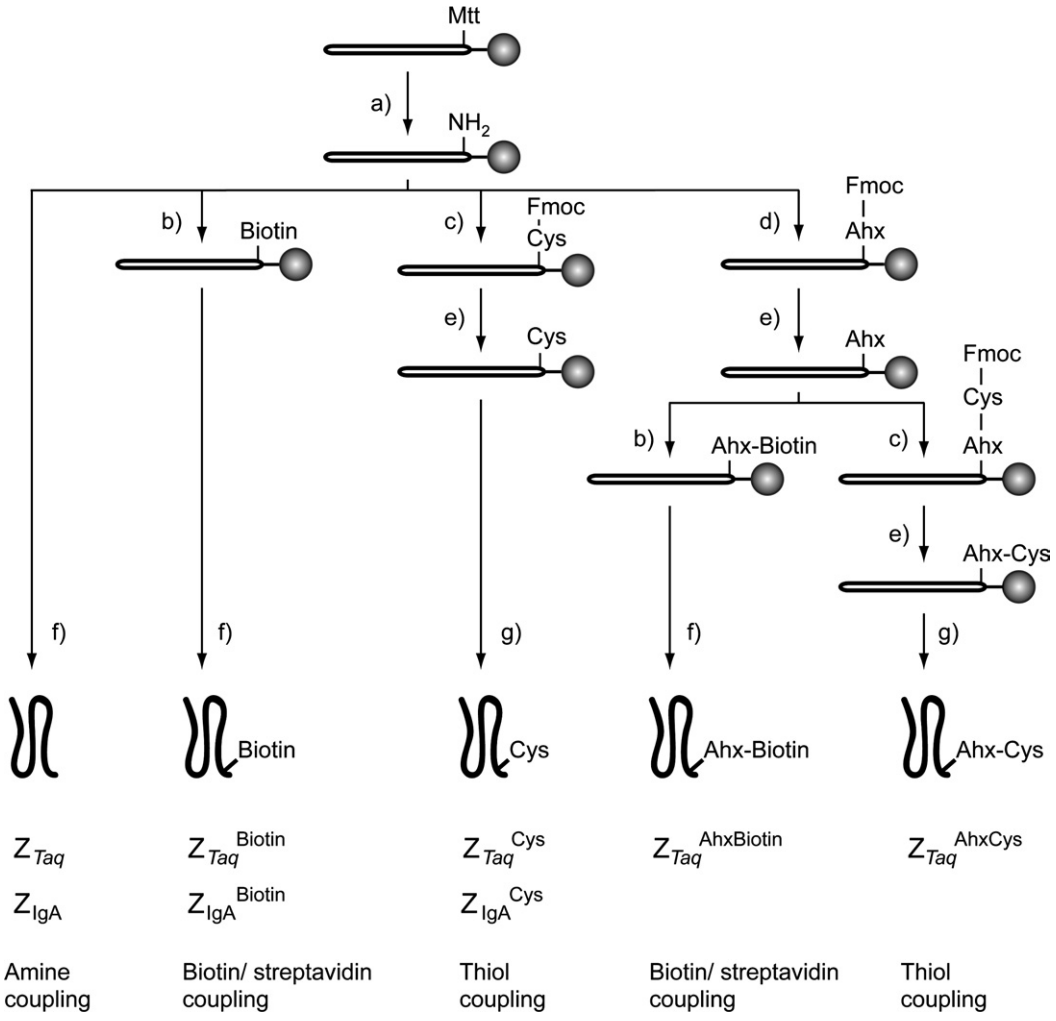

Fig. 1. Synthetic scheme for the preparation of five different affibody variants: (a) TFA:TIS:DCM (1:5:94), 10×2 min; (b) D-biotin, HBTU, HOBt, DIEA in NMP, 2×30 min; (c) Fmoc-Cys(Trt)-OH, HBTU, HOBt, DIEA in NMP, 2×30 min; (d) Fmoc-Ahx-OH, HBTU, HOBt, DIEA in NMP, 2×30 min; (e) 20% piperidine–NMP, 20 min; (f) TFA:TIS:H_2O (95:2.5:2.5), 3 h; (g) TFA:TIS:EDT:H_2O (94:1:2.5:2.5), 3 h.

Manual modification

The resin-bound peptides were manually modified by the introduction of thiol and biotin groups for selective surface immobilization (Fig. 1). The Mtt protection of the side chain of Lys^{58} was removed by 10×2 min treatment of the peptide resin with TFA:TIS:dichloromethane (DCM) (1:5:94). D-Biotin, Fmoc-Cys(Trt)-OH, or Fmoc-Ahx-OH was coupled to the free amino group with HBTU/HOBt/DIEA for 30 min. The efficiency of the coupling reaction was monitored by a ninhydrin test, and the coupling was repeated if necessary. The Fmoc group was removed from Fmoc-Cys(Trt) and Fmoc-Ahx by 20 min treatment with 20% piperidine–NMP. D-Biotin or Fmoc-Cys(Trt)-OH was then coupled, as described above, to the affibody variants modified with the Ahx spacer. Finally, the Fmoc protecting group was removed from Fmoc-Cys(Trt)-Ahx, as described above, prior to cleavage from the resin.

Final cleavage and deprotection

Cleavage was performed in TFA:TIS:H_2O (95:2.5:2.5) for peptides not containing cysteine and in TFA:TIS: ethanedithiol (EDT):H_2O (94:1:2.5:2.5) for peptides containing cysteine. The cleavage reaction was allowed to proceed for 3 h at room temperature. The reaction mixture was then extracted three times with *tert*-butylmethyl ether and water, followed by filtration and lyophilization of the water phase.

Purification and analysis of the synthetic proteins

The synthetic affibody molecules were purified by reverse-phase HPLC using a 4.6×150-mm column with a polystyrene/divinyl benzene matrix and 5 μm particle size (Amersham Biosciences, Uppsala, Sweden). A flow rate of 1 ml min^{-1} and an elution gradient of 30–40% B in 25 min, where solvent A was 0.1% TFA–H_2O and

solvent B was 0.1% TFA–CH_3CN, was used for the purification of all protein variants. Eluted fractions were lyophilized, and part of each fraction was dissolved at a concentration of 1 μM in H_2O:CH_3CN (1:1) containing 0.1% HCOOH and injected into a positive mode ESI Q-TOF 2 mass spectrometer (Micromass, Manchester, UK) to verify the correct mass of the purified protein. The remaining protein was dissolved in phosphate-buffered saline (PBS, pH 7.4), and the protein was analyzed by 20% homogeneous SDS–PAGE on a Phast system (Amersham Biosciences) to confirm the purity and estimate the concentration by comparison with a protein standard of known concentration. Protein concentration was also determined through absorption measurements at 280 nm and calculation of the concentration from the extinction coefficient (www.expasy.org).

Target protein production and labeling

The *Taq* DNA polymerase fusion protein (108 kDa) used as the target protein contained a divalent version of the IgG-binding Z domain [21] fused to an extension of *Taq* DNA polymerase described earlier [22]. The fusion protein was produced intracellularly in *Escherichia coli* using a trp promoter-based expression system [23] and was purified by IgG affinity chromatography. Human IgA from colostrum was purchased from Sigma–Aldrich (cat. no. I-1010). IgA is known to exist in both monomeric and different polymeric forms, but for simplicity a molecular weight of 160 kDa [24] was used for the calculations in this study. For the microarray assays, 1 mg of IgA and *Taq* DNA polymerase was labeled with one tube of Cy3 (Cy3 Mono-reactive Dye Pack, Amersham Biosciences, Buckinghamshire, UK) in PBS (pH 7.4) for 1–2 h, after which excess dye was removed by gel filtration on a NAP10 column (Amersham Biosciences, Uppsala, Sweden). Labeled target protein was stored refrigerated until use.

SPR assays

Affibody immobilization on biosensor slides

The affibody variants Z_{Taq}, Z_{IgA}, Z_{Taq}^{Cys}, Z_{Taq}^{AhxCys}, and Z_{IgA}^{Cys} were immobilized according to the supplier's recommendations onto the carboxymethylated dextran layer of CM5 sensor chips (Biacore, Uppsala, Sweden) at a relative response of 100–300 response units (RU). The unmodified proteins were immobilized by amine coupling in 2 mM NaOAc (pH 4.5) to a sensor chip activated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) in water. The proteins modified with cysteine were immobilized in 2 mM NaOAc (pH 4.5) by thiol–disulfide exchange with a reactive disulfide group introduced to the sensor chip by activation with EDC/NHS in water, followed by reaction with 80 mM 2-(2-pyridinyldithio)ethaneamine (PDEA) in 0.1 M $Na_2B_4O_7$ (pH 8.5). Streptavidin SA sensor chips (Biacore) were pretreated with three injections of 5 μl of 1 M NaCl + 50 mM NaOH, followed by five injections of 5 μl of 0.05% SDS, before immobilization of Z_{Taq}^{Biotin}, $Z_{Taq}^{AhxBiotin}$, and Z_{IgA}^{Biotin} in HBS buffer (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4) at 100–300 RU.

Biospecific interaction analysis

Taq DNA polymerase (30 μl) at three different concentrations (100, 410, and 1000 nM) was injected in duplicate on two separate sensor chips, and the response was monitored on a Biacore 2000 instrument. All experiments were run at a flow rate of 5 μl min^{-1} with HBS as the running buffer. Between injections, the surfaces were regenerated by injection of 5 μl of 0.05% SDS. The nonspecific binding to a control surface (Z_{IgA}) was subtracted from the response. The specific binding activity of the affibody variants was calculated using the equation.

$$\text{Specific activity}(\%) = \frac{RU_{Taq}}{RU_{Affibody}} \times \frac{MW_{Affibody}}{MW_{Taq}} \times 100, \quad (1)$$

where RU_{Taq} is the specific response to the injected *Taq* DNA polymerase after subtraction of the negative control, $RU_{Affibody}$ is the response from the immobilization of the affibody, and $MW_{Affibody}$ and MW_{Taq} are the molecular weights of the respective proteins. All Biacore runs were repeated on a second slide, and the mean of the two slide runs and the standard deviation were calculated.

Microarray slide preparation

Carboxymethyl dextran slides

The carboxymethyl dextran (CMD) slides (XanTec Bioanalytics, Muenster, Germany) were washed with water once or twice for 10–30 min, followed by the addition of an activation mixture consisting of 0.3 M EDC (Bachem, Bubendorf, Switzerland) and 0.5 M NHS (Bachem) in 0.5 M 2-morpholinoethanesulfonate (MES) buffer (pH 5.0) (BDH Laboratory Supplies, Poole, UK). The slides were activated for 7–15 min. The activated slides were washed twice with 2 mM acetic acid, washed once with water, and spun dry on a Minicentrifuge (Merck Eurolab, Stockholm, Sweden). The slides were spotted within 3 h.

Thiol dextran slides

The thiol dextran (TD) slides (XanTec Bioanalytics) were washed with water once or twice for 10–30 min and treated with 100 mM DTT (Sigma–Aldrich) in 0.1 M Na_2PO_4 (pH 8.1) for 20 min to reduce the disulfide bonds on the slide surface. The slides were then

washed once with water, followed by activation through a 20-min treatment with 10 mM 2,2′-pyridyl disulfide (Lancaster Synthesis, Morecambe, UK) in 0.1 M Na_2PO_4 (pH 8.1) containing 20% ethanol. After activation, the slides were washed three to five times with water and spun dry. The slides were spotted within 3 h.

Xenoslide S streptavidin-coated microscope slides

The Xenoslide S streptavidin-coated microscope slides were ready for use on arrival (Xenopore, Hawthorne, NJ, USA).

Microarray slide spotting

Synthetic affibody molecules were dissolved at a concentration of 1 mg ml^{-1} in 2 mM NaOAc (pH 4.5) for spotting onto CMD slides and TD slides and in 1× PBS (pH 7.4) for spotting onto streptavidin slides. A total of 32 arrays were printed on each slide with a GMS 427 arrayer (Affymetrix, Santa Clara, CA, USA). Each array contained three replicates of the affibody variants: Z_{Taq} and Z_{IgA} for CMD slides; Z_{Taq}^{Cys}, Z_{Taq}^{AhxCys}, and Z_{IgA}^{Cys} for TD slides; and Z_{Taq}^{Biotin}, $Z_{Taq}^{AhxBiotin}$, and Z_{IgA}^{Biotin} for streptavidin slides. All slides were incubated in 70–80% humidity at room temperature overnight after printing and were subsequently blocked in Superblock Dry Blend (TBS, Boule Nordic, Huddinge, Sweden) for 3–4 h at room temperature. The slides were then spun dry, stored air-tight, and refrigerated with silica gel until use.

Target protein incubation, microarray slide scanning, and evaluation

All slide types were incubated overnight at room temperature with 30 μl Cy3-labeled IgA or *Taq* DNA polymerase diluted in 1× PBS (pH 7.4), supplemented with 0.25% casein from bovine milk (Sigma–Aldrich), at concentrations ranging from 3 pM to 100 nM. A 384-well silicon mask with an in-house fabricated mask holder, using four screws to hold the mask in place and prevent leakage, was used to separate the individual arrays. Target protein was added to each array, with every second array left empty to minimize the risk of cross-contamination. After overnight incubation, each array of the dilution series was washed twice with 2× PBS (pH 7.4). The mask was then opened in 2× PBS (pH 7.4), and the slide was washed another 3 × 5 min in 2× PBS (pH 7.4) before being spun dry on a minicentrifuge. All slide experiments were performed in duplicate on two different slides. Slides were scanned with an Agilent Scanner (Agilent Technologies, Paramus, NJ, USA) using the green channel. The 16-bit output TIFF images were imported into GenePix 5.0 (Axon Instruments, Union City, CA, USA). The median local background was subtracted from the median signal of the spots to give the relative fluorescence signal, and a mean was calculated from the triplicates of each concentration and protein. A new mean with interslide standard deviation was calculated from the two triplicates of two independent slides. Limit of detection was defined as the lowest concentration of target protein where the signal from the target-specific affibody was higher than the mean blank (the control affibody of other binding specificity) plus 1.96 standard deviation (95% confidence interval).

Results

The Z_{Taq} and Z_{IgA} affibody molecules were synthesized on an automated peptide synthesizer, followed by further modification of parts of the peptide resin by manual synthesis (Fig. 1). Unmodified proteins were used for random immobilization by amine coupling through the N-terminal α-amino group and the lysine side chain ε-amino groups. Two protein variants for oriented immobilization by thiol coupling or biotin–streptavidin coupling were prepared by the coupling of cysteine or biotin to the side chain of the C-terminal lysine residue (Lys^{58}) in Z_{Taq} and Z_{IgA}. For Z_{Taq}, two additional constructs were prepared by the incorporation of a spacer (Ahx) between the affibody and the cysteine or biotin moiety.

Affibody synthesis

The typical yield after synthesis of the 58-amino acid proteins was 30%, and after manual modification it was 20%. This corresponds to an average efficiency of 98% per cycle for the synthesis of the full-length protein and a yield of 70% for the on-resin protein modification. After HPLC purification, the masses of the synthetic proteins were analyzed with MS (data not shown), verifying that the correct affibody molecule derivatives had been prepared.

SPR analysis

As a first evaluation of the different immobilization strategies, the five synthetic Z_{Taq} variants (Fig. 1) were immobilized on biosensor chips using different coupling chemistries, and the specific activities of the affibody molecules were determined by SPR. *Taq* DNA polymerase at three different concentrations (100, 410, and 1000 nM) was injected over sensor chip surfaces prepared with the affibody variants. The mean responses and standard deviations were calculated from two biosensor chips (Fig. 2). Although the aim was to immobilize equal amounts of all affibody capture proteins on the sensor slides, it was noted that immobilization at the lower protein density in the obtained range (100–300 RU) led to slightly more variation in the response

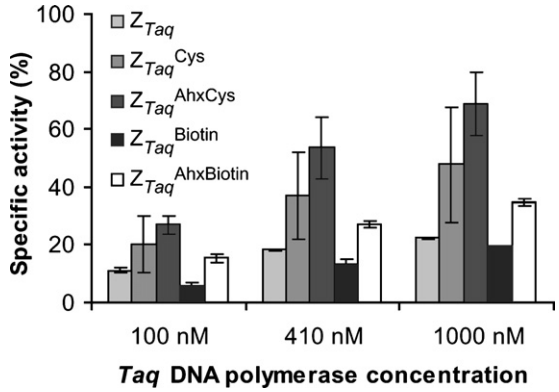

Fig. 2. Column chart showing the specific activity of five different affibody variants when subjected to three concentrations of *Taq* DNA polymerase (100, 410, and 1000 nM) in the Biacore biosensor analysis. Z_{Taq} was immobilized by amine coupling to CMD slides, Z_{Taq}^{Cys} and Z_{Taq}^{AhxCys} were immobilized by thiol coupling to TD slides and Z_{Taq}^{Biotin} and $Z_{Taq}^{AhxBiotin}$ were immobilized onto streptavidin-coated slides.

on binding of *Taq* DNA polymerase, and this can explain the relatively large difference in standard deviations for the different affibody constructs.

The Z_{Taq} variant, immobilized randomly by amine coupling using EDC/NHS chemistry, gave a specific concentration-dependent response when exposed to *Taq* DNA polymerase. The Z_{Taq}^{Cys} variant, immobilized by oriented coupling using thiol–disulfide exchange with the PDEA group, had higher specific activity than Z_{Taq} for all concentrations of injected target protein. Even higher specific activity was achieved for Z_{Taq}^{AhxCys}, where an aminohexanoic acid spacer placed the affibody farther away from the chip surface. However, oriented immobilization of Z_{Taq}^{Biotin} through the high-affinity interaction between biotin and streptavidin resulted in lower specific activity of the affibody than did randomly oriented unmodified Z_{Taq}. The addition of a spacer between the affibody and the biotin moiety in $Z_{Taq}^{AhxBiotin}$ improved the specific activity. However, the $Z_{Taq}^{AhxBiotin}$ variant still had lower specific activity than Z_{Taq}^{Cys}. In conclusion, directed immobilization of Z_{Taq}^{AhxCys} through thiol coupling gave the highest specific activity of all immobilization techniques evaluated by SPR.

Microarray experiments

Following the SPR experiments, the same immobilization chemistries were used for coupling the affibody molecules to microarray slides. The proteins were spotted using a solid pin contact arrayer based on the pin and ring technology. A prestudy was first carried out to determine the signal intensities of different concentrations of affibody molecules spotted on microarray slides and incubated with a fixed concentration of target protein. An increase in spot signal intensity was detected with higher concentrations of spotted affibody over the concentration span 0.25–1 mg ml^{-1} (data not shown).

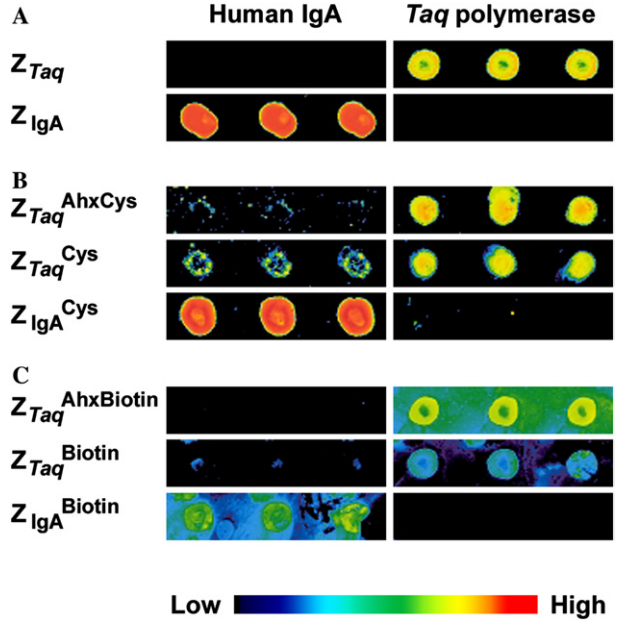

Fig. 3. Typical GenePix images of the affibody spots on (A) CMD slides, (B) TD slides, and (C) streptavidin slides. The slides were scanned after incubation with 10 nM of human IgA (left panel) or 9 nM of *Taq* DNA polymerase (right panel). Affibody constructs are shown spotted in triplicate. All spots for each slide type were excised from the same slide and from the same arrays for the two target proteins.

Because 1 mg ml^{-1} gave the highest relative fluorescence, the microarray slides used in the following experiments were spotted with this concentration of affibody molecules.

The microarray slides were spotted with affibody molecules, incubated with Cy3-labeled *Taq* DNA polymerase or Cy3-labeled human IgA overnight, washed, and scanned. No leakage or cross-contamination between arrays could be observed, and all spots had a similar shape and size with a local signal decrease where the pin had touched the slide surface (Fig. 3). Signal intensities were similar among array triplicates. Following fluorescent scanning and image analysis, a mean of the signal from the two triplicates from two independent slides was calculated, and the target concentration-dependent signals were plotted with interslide standard deviation error bars (Fig. 4). Inset graphs in the Fig. 4 panels show the signals at the concentrations calculated as the limit of detection.

CMD slides

Random immobilization of affibody molecules onto the CMD surfaces gave low slide background signals and high relative fluorescence (Fig. 3A). For both variants, no cross-reactivity with the control protein was observed, showing that the affibody molecules had high selectivity for binding human IgA and *Taq* DNA polymerase, respectively. A concentration-dependent in-

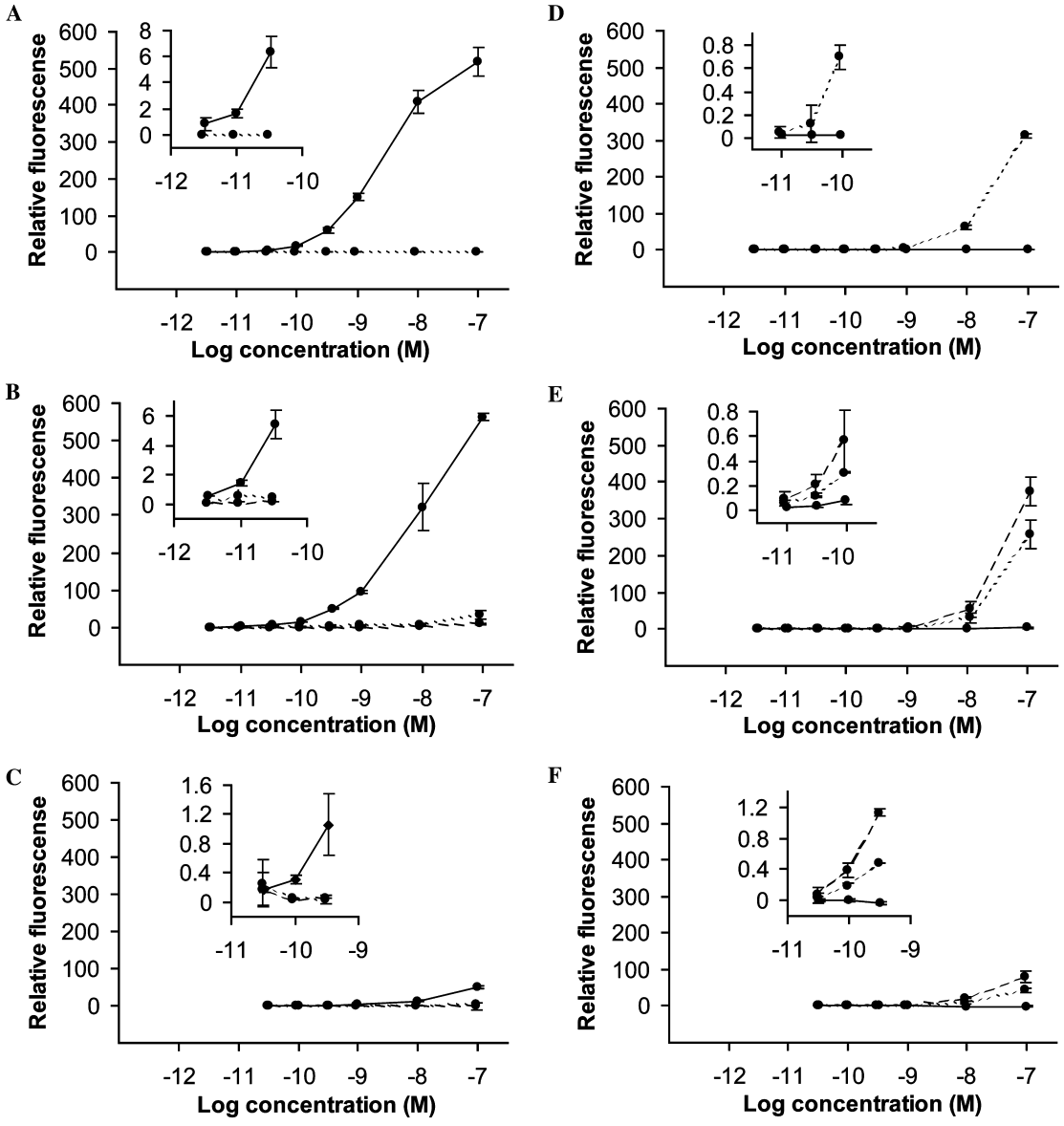

Fig. 4. Relative fluorescence of Z_{Taq} and Z_{IgA} spots on microarray slides, incubated with increasing concentrations of Cy3-labeled human IgA (A–C) or Cy3-labeled *Taq* DNA polymerase (D–F). An inset graph in each panel shows the signal at the concentration calculated as the limit of detection. (A,D) Z_{Taq} (- - -) and Z_{IgA} (—) immobilized by amine coupling to CMD slides. (B,E) Z_{Taq}^{Cys} (- - -), Z_{Taq}^{AhxCys} (– –), and Z_{IgA}^{Cys} (—) immobilized by thiol coupling to TD slides. (C,F) Z_{Taq}^{Biotin} (- - -), $Z_{Taq}^{AhxBiotin}$ (– –), and Z_{IgA}^{Biotin} (—) immobilized onto streptavidin slides.

crease in the fluorescent signal on incubation with increasing concentrations of target protein was observed for the concentration span 3 pM to 100 nM (Figs. 4A and D). The limits of detection were 3 pM for human IgA (Fig. 4A) and 90 pM for *Taq* DNA polymerase (Fig. 4D).

TD slides

Directed immobilization through cysteine on TD surfaces gave results comparable to those of random immobilization on the CMD surfaces (Fig. 3B). The slide background was low, and the relative fluorescence was high. A low level of binding to the control affibody spots could be observed; however, because no cross-reactivity of the affibodies was observed on the CMD slides, the binding to the spots on the TD slides was probably due to disruption of the thiol surface by the contact with the pins or by affibody carryover resulting from insufficient washing of the pins during the spotting procedure. The limits of detection were 10 pM for human IgA (Fig. 4B) and 30 pM for *Taq* DNA polymerase (Fig. 4E), values that are comparable to those obtained with the CMD slides. The addition of a spacer between the affibody and the cysteine residue gave higher relative fluorescence over the entire concentration span for *Taq* DNA polymerase.

Streptavidin slides

Directed immobilization through biotin was carried out using streptavidin slides (Fig. 3C). The streptavidin surfaces gave a high and uneven background compared with the CMD and TD slides and also showed a four times lower relative fluorescence at the highest concentration. The slide surface background was particularly high in the regions proximal to the affibody spots involved in binding the specific target protein, suggesting a contribution from protein dissociated from the spots during the incubation and washes. The limits of detection were 100 pM for human IgA (Fig. 4C) and 90 pM for *Taq* DNA polymerase (Fig. 4F). The addition of a spacer between the affibody and the biotin moiety gave a higher relative fluorescence over the entire concentration span of *Taq* DNA polymerase, consistent with the improved results obtained by the incorporation of a spacer for coupling to the TD slides.

Discussion

The small size of the affibody scaffold (58 amino acids) makes it readily available to synthesis by standard Fmoc solid phase peptide chemistry, and in the current study chemical synthesis was used to prepare affibody molecules with different site-specific modifications for directed coupling to microarray surfaces. The immobilization chemistries were first evaluated in the SPR-based Biacore system, where the response that is recorded is directly proportional to the mass of the molecules that are bound to the biosensor surface. Because both the amount of the immobilized capture protein and the bound target protein can be directly monitored in the Biacore system, the specific activity of the affibody molecules immobilized on the surface by different immobilization chemistries could be determined. In the second part of the study, affibody molecules were immobilized by different coupling strategies on microarray slides, and the overall suitability of the different slide types for the production of affibody protein capture arrays was evaluated.

In the Biacore system, the affibody was shown to be functional on all sensor slide surfaces (Fig. 2). Z_{Taq}^{Cys} immobilized by thiol coupling had higher specific activity than did Z_{Taq} randomly immobilized by amine coupling, indicating that the affibody is more accessible to binding when an oriented immobilization is used. For the amine coupling, the sensor slide was activated with EDC and NHS to form an active ester, which could react with the N-terminal α-amino group or the ε-amino group in the side chain of any of the six lysine residues in Z_{Taq} [19]. Although the α-amino group is expected to be the most reactive species at the low pH (4.5) used in the coupling reaction, ε-amino groups will probably also react with the surface. As a result, a heterogeneous slide surface with affibody molecules in different orientations and with multiple attachment points, where some of the proteins are not available for binding, is likely formed. In contrast, thiol coupling through thiol–disulfide exchange of the cysteine residue in Z_{Taq}^{Cys} with a reactive disulfide introduced by the coupling of a PDEA moiety to the sensor slide should potentially produce a homogeneous sensor surface with all of the affibody molecules in the same orientation. In the synthesis of the affibody construct used for directed thiol coupling, the cysteine residue was attached to the ε-amino group of the side chain of Lys^{58}, which is the C-terminal residue in the protein. This position was chosen because it is located outside the helical region of the parental scaffold [9,25] and would presumably lead to efficient presentation of the randomized binding residues in helices 1 and 2 to the surrounding medium. The results indicate that this was a valid approach because the affibody immobilized by directed thiol coupling through the side chain of Lys^{58} gave a higher response than did the randomly immobilized affibody (Fig. 2). The addition of a six-carbon spacer in Z_{Taq}^{AhxCys} resulted in even higher specific activity, showing that by increasing the distance between the affibody and the chip surface, the access of the affibody to the target protein could be further improved. However, directed immobilization of Z_{Taq}^{Biotin} to a streptavidin-coated slide gave lower specific activity than did random immobilization of unmodified Z_{Taq} when studied in the Biacore system, indicating that the biotinylated affibody bound to streptavidin is not fully accessible to binding its target protein. After the addition of a six-carbon spacer in $Z_{Taq}^{AhxBiotin}$, the specific activity was increased, consistent with the hypothesis that the spacer makes the affibody more accessible to binding its target protein.

In the second part of the study, the performance of the affibody molecules as capture agents on different types of microarray slides was evaluated. In agreement with the results of the Biacore studies, it was expected that oriented immobilization of the affibody molecules would also be the preferred strategy for the preparation of microarray slides. However, when spotting the affibody molecules Z_{IgA} and Z_{Taq} to CMD slides (Figs. 4A and D) and spotting Z_{IgA}^{Cys}, Z_{Taq}^{Cys}, and Z_{Taq}^{AhxCys} to TD slides (Figs. 4B and E), followed by incubation with fluorescent-labeled target protein, similar relative fluorescence signals and detection limits were recorded for both types of slides. Although all microarray slides were spotted with the same concentration of protein (1 mg ml^{-1}), it cannot be excluded that the efficiencies of the amine coupling and thiol coupling were not the same and that different amounts of capture protein were immobilized on the CMD slides and TD slides. Therefore, it is possible that the specific activity of the affibody immobilized in a directed manner was higher than that of the randomly immobilized affibody but that a lower

amount of affibody was present on the chip surface. However, when considering the combined effect of the protein coupling efficiency and the specific activity of the immobilized protein for slide preparation, the performance of the two slide types was found to be similar.

Another important factor affecting the sensitivity and reproducibility of the microarray experiments is the background signal, which to a large extent is determined by inherent properties of the microarray surface and the degree of nonspecific binding. The streptavidin slides used for spotting Z_{IgA}^{Biotin}, Z_{Taq}^{Biotin}, and $Z_{Taq}^{AhxBiotin}$ showed very high background staining in areas surrounding the spots (Fig. 3C), and they also showed lower relative fluorescence signals and higher detection limits (Figs. 4C and F). In the CMD slides and TD slides, the proteins are coupled to functional groups linked to a dextran layer, which is efficient in suppressing nonspecific interactions [26]. However, streptavidin is coated directly onto the glass slides in the production of streptavidin slides, and the lack of a hydrogel coating could possibly explain the higher background signal. In addition, it is likely that the proteins are immobilized at a lower density on the streptavidin slides than on the dextran-coated slides because the hydrogel coating produces a larger solvent-exposed surface area to which the proteins can be coupled, and this could be a contributing factor to the observed difference in limit of detection.

Earlier studies on the immobilization of Fab fragments and complete antibodies on microarray slides showed that higher relative signals were achieved when the ligands were immobilized in an oriented fashion [17]. In the current study, random immobilization and directed immobilization worked equally well for immobilization onto microarray slides. Amine coupling is the simplest method because no additional modifications must be introduced into the binding protein for coupling to the microarray surface; therefore, it may be the first choice for production of affibody microarrays. However, amine coupling is not ideal for ligands where lysine residues are important for the binding interaction, and for these proteins thiol coupling is a good alternative. The experiments suggest that there is flexibility in the choice of slide type for the production of affibody microarrays because all of the tested coupling chemistries could be used to produce slides where the two affibody molecules in the study were functional and could bind their respective target proteins with high specificity.

As shown by the calculated limit of detection, the sensitivity of the affibody capture microarrays is sufficient for most purposes, but for the analysis of low-abundance proteins, it might be necessary to increase the signal. Interestingly, in the microarray experiments, the limit of detection for IgA was lower than that for *Taq* DNA polymerase, although the dissociation constant (K_d) for Z_{IgA} binding to IgA is 0.5 μM [12], whereas the K_d for Z_{Taq} binding to *Taq* DNA polymerase is 25 nM [19]. It is likely that the low limit of detection for IgA is caused by an avidity effect because it is possible that two binding sites for Z_{IgA} are available on the symmetrical IgA molecule, and this would allow two affibody molecules to bind one IgA molecule simultaneously and contribute to a higher apparent affinity. Higher aggregates of the IgA molecule are known to form in vivo, and this could further accentuate the avidity effects.

In conclusion, the affibody molecules Z_{Taq} and Z_{IgA} were successfully immobilized onto biosensor and microarray slides by amine coupling, thiol coupling, and biotin–streptavidin coupling with retained function and binding specificity. Oriented immobilization through a cysteine residue attached to the side chain of the C-terminal residue in the affibody was shown to give the highest specific activity in the Biacore experiments. In the microarray experiments, oriented immobilization by thiol coupling did not enhance, as expected, the results of the random immobilization by amine coupling. Noncovalent coupling of the affibody through the biotin–streptavidin coupling gave lower specific activity in the Biacore system and high surface background and a lower relative fluorescence signal on the microarray slides.

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