

Antibody Arrays Prepared by Cutinase-Mediated Immobilization on Self-Assembled Monolayers

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Antibody arrays hold considerable potential in a variety of applications including proteomics research, drug discovery, and diagnostics. Many of the schemes used to fabricate the arrays fail to immobilize the antibodies at a uniform density or in a single orientation; consequently, the immobilized antibodies recognize their antigens with variable efficiency. This paper describes a strategy to immobilize antibodies in a single orientation, with a controlled density, using the covalent interaction between cutinase and its suicide substrate. Protein fusions between cutinase and five antibodies of three different types (scFv, V_{HH}, and FN3) were prepared and immobilized upon self-assembled monolayers (SAMs) presenting a phosphonate capture ligand. The immobilized antibodies exhibit high affinity and selectivity for their target antigens, as monitored by surface plasmon resonance and fluorescence scanning. Furthermore, by changing the density of capture ligand on the SAM the density of the immobilized antibody could be controlled. The monolayers, which also present a tri(ethylene glycol) group, are inert to non-specific adsorption of proteins and allow the detection of a specific antigen in a complex mixture. The demonstration of cutinase-directed antibody immobilization with insert SAMs provides a straightforward and robust method for preparing antibody chips.

Protein array technology has emerged as a promising tool for screening protein–protein interactions¹ and characterizing the levels of proteins expressed in cells.² In particular, antibody arrays are being widely developed as tools for profiling the proteins of a

cell, with applications for understanding cellular responses to various stimuli and discovering protein markers of disease.³ The preparation of an antibody array requires immobilization of immunoglobulins to a solid support. The simplest immobilization methods rely on noncovalent adsorption of antibodies onto glass surfaces coated with poly-L-lysine^{4a,b} or nitrocellulose membranes^{4c} or covalent attachment to surfaces chemically modified with aldehydes, activated esters, maleimides, or epoxide cross-linkers.^{4d} While both types of immobilization strategies are simple—they do not require biochemical or chemical modification of proteins—they generally suffer from antibody loss due to denaturation, nonspecific adsorption of proteins, adsorption in inactive orientations, and poor control over the densities of the immobilized proteins.⁵ In addition, both methods require that the antibodies must be purified prior to immobilization.

Methods that immobilize antibodies in a single orientation, with the antigen-binding site positioned away from the surface and facing the solution, would optimize the activities of immobilized antibodies and the performance of arrays.⁶ One common strategy involves the chemical biotinylation of antibodies, followed by their subsequent immobilization onto avidin- or streptavidin-coated surfaces.⁷ While biotinylated antibodies are likely to retain their native conformation when immobilized, the antibodies are linked in multiple orientations relative to the surface, because the chemical addition of biotin is not at a single site. This drawback can be addressed through strategies that allow a site-specific

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introduction of biotin, which in turn provide a uniform orientation of the immobilized antibody.⁷ Alternatively, oriented immobilization of an antibody can be accomplished by first immobilizing an intermediate protein (i.e., protein A,^{8a,b} protein G,^{8c} Fc-binding antibody^{8d-f}) that binds to the heavy chain constant (Fc) region of antibodies. However, the need for two protein immobilization steps can result in a low density of the immobilized antibody.^{7,8}

A separate challenge in preparing antibody arrays concerns the availability of useful antibodies with the appropriate affinity and specificity for their antigens. Indeed, many attempts to prepare antibody arrays for a panel of targets or analytes have required substantial effort in developing the antibodies.⁹ Such limitations may be addressed with phage, yeast, ribosome, and RNA display technologies,¹⁰ which offer a rapid route for generating affinity reagents for new proteomic targets. These affinity reagents include single-domain antibodies (V_{HH}),¹¹ single-chain fragments of variable regions (scFv),¹² fragments of antigen binding (Fab), and various protein scaffolds that can be engineered for binding to biomolecules (i.e., fibronectin type III domain, lipocalin, and helix-bundle structures).^{13,14} Several advantages of the engineered proteins, as compared to monoclonal antibodies, include smaller size, higher levels of expression in *Escherichia coli*, and the ability to engineer stability, selectivity, and affinity.¹⁵ These properties make the affinity reagents attractive substitutes for monoclonal antibodies in the fabrication of protein and antibody arrays. (Throughout the paper, we use the general term "antibody" in the broad sense to include engineered affinity reagents such as antibody fragments, domain antibodies, and artificial antibodies.) We show that the cutinase-mediated immobilization strategy works well for each of these classes of affinity reagents.

In order to develop a simple and general approach for fabricating antibody arrays, we have adopted the recently de-

scribed cutinase-mediated method to immobilize antibodies.¹⁶ Cutinase is a 22 kDa serine esterase that forms a site-specific covalent adduct with phosphonate ligands through the nucleophilic attack of a catalytic serine residue (Ser 120) on the electrophilic phosphonate. Thus, to immobilize an antibody by this approach, we first prepared a fusion of the antibody and cutinase proteins and then captured the fusion proteins on SAMs presenting the phosphonate ligand (Figure 1A). Herein, we illustrate this strategy and use surface plasmon resonance (SPR) spectroscopy and fluorescence methods to characterize the immobilization of antibodies and their selective binding to antigens.

MATERIALS AND METHODS

Materials and Instrumentation. Chemicals and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification. Phosphate-buffered saline (PBS) was purchased from GibcoBRL (Carlsbad, CA). Surface plasmon resonance (SPR) spectroscopy was performed with the Biacore 1000 (Biacore, Uppsala, Sweden), and fluorescence arrays were scanned in a GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA) using a 532-nm wavelength laser source.

Construction of the Expression Vector. The pCutΔEZ plasmid¹⁶ was modified in the following manner. Between the multiple cloning sites and the cutinase encoding sequence, a flexible (GGGG)₃ linker was incorporated by inserting the annealed synthetic oligonucleotides (5'-gat ctg gcg gtg gcg gtt ctg gtg gcg gtg gct ctg gtg gtg gtt ctg gat ccg-3' and 5'-aat tcg gat cca gaa cca cca cca gag cca ccg cca cca gaa ccg cca ccg cca-3') between the *EcoR* I and *Bam*H I sites of pCutΔEZ to yield the modified plasmid pCut2. Gene fragments were prepared by polymerase chain reaction (PCR) to encode the single-chain fragments of the antibody variable region (scFv) that recognize the SH3 domain of human c-Src and the N-terminal EH domain of frog intersectin, the variable domain of a camel heavy chain (V_{HH}) antibody that recognizes hen lysozyme, and the engineered 10th fibronectin type III domains (FN3/monobody) that bind the Src SH3 domain and streptavidin.¹⁷ The prepared gene fragments were inserted between the *Bam*H I and *Hind* III sites of the pCut2 plasmid. The constructs were confirmed by DNA sequencing and transformed into *Escherichia coli* BL21 (DE3) (Novagen, Madison, WI) via electroporation. Expression of the cutinase fusion proteins was driven by a T7 RNA polymerase promoter, and expressed proteins were directed to the periplasmic space by the preceding pelB signal peptide. A six-histidine tag at the C-terminus of the cutinase fusion proteins was used for purification by immobilized metal affinity chromatography (IMAC).

Expression and Purification of the Cutinase Fusions. Recombinant *E. coli* BL21(DE3) cells, harboring the corresponding constructs, were inoculated into Luria-Bertani (LB) medium containing carbenicillin (50 μg/mL) and incubated overnight at 37 °C with shaking at 250 rpm. The overnight culture (5 mL) was transferred into fresh LB medium (1 L) with carbenicillin and incubated until an OD_{600 nm} of 0.5 was reached, at which time isopropyl-α-D-thiogalactopyranoside (IPTG) was added to a final

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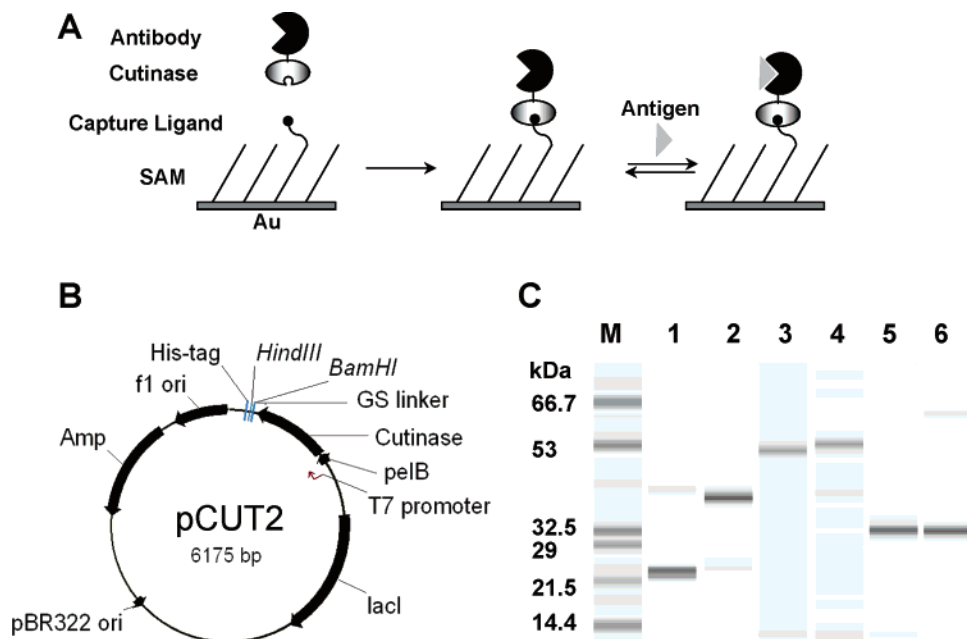


Figure 1. Strategy for immobilization of antibodies. (A) Antibodies were prepared as cutinase fusions and immobilized to SAMs presenting a phosphonate capture ligand through a covalent interaction between cutinase and the capture ligand. Antigens were applied to immobilized antibodies to monitor the antigen–antibody interaction. (B) Expression of cutinase and its fusions. Gene fragments encoding cutinase and its fusions were cloned into the pCUT2 plasmid. The resulting recombinant plasmids were introduced into *E. coli* origami B (DE3) strains (cuti- V_{HH} and cuti-scFv) or *E. coli* BL21 (DE3) strains (cuti-FN3) to express proteins. (C) The expressed proteins were purified under either native or denaturing conditions and refolded on a column as described. The purity and molecular weight of the purified and refolded proteins were analyzed on an Agilent Bioanalyzer 2100. Molecular weight standards (M) were used, and cutinase, cuti- V_{HH} /lysozyme, cuti-scFv/EH, cuti-scFv/Src SH3, cuti-FN3/Src SH3, and cuti-FN3/streptavidin are shown in lanes 1–6, respectively. Molecular weights are noted as kilodaltons (kDa).

concentration of 0.3 mM. The culture was incubated an additional 16 h at 18 °C (30 °C for cuti-FN3 fusions), with shaking at 250 rpm. Cells were collected by centrifugation at 4000g for 20 min. Cutinase was prepared as described previously.¹⁶ Cutinase-fused FN3 domains were purified from *E. coli* cell extracts by IMAC under native conditions according to the vendor's protocol (Qiagen, Valencia, CA).¹⁷ Since the cutinase fusions with scFv and V_{HH} were expressed as insoluble aggregates, these proteins were purified under denaturing conditions and refolded on the column.¹⁸ Briefly, the bacterial cells were lysed by sonication and the insoluble fraction was recovered by centrifugation at 8000g for 20 min. Lysis buffer (100 mL, pH 8.0, 10 mM Tris–HCl, and 100 mM NaH_2PO_4) containing 8 M urea was added to solubilize the pellet, generated from a 1 L culture, and the solution was incubated at 4 °C with stirring for 3 h. An equal volume of washing buffer (pH 8, 50 mM NaH_2PO_4 , 300 mM NaCl, and 10 mM imidazole) was added to the solubilized protein in the presence of reduced glutathione (GSH, 3 mM) and oxidized glutathione (GSSG, 0.3 mM). The mixture was incubated at 4 °C for 6 h before aggregates were removed by centrifugation at 8000g for 30 min. The supernatant was incubated with Ni–NTA agarose (6 mL) at room temperature for 2 h and then applied to the column. The loaded column was washed stepwise with 50 mL of 4 M, 3 M, 2 M, and 1 M urea, and finally with 75 mL of wash buffer. The His-tagged fusion proteins were eluted with native elution buffer (50 mM NaH_2PO_4 , 300 mM NaCl, and 250 mM imidazole). The eluted

proteins were dialyzed against PBS to remove imidazole at 4 °C and profiled for quantity, molecular weight, and purity using a BioAnalyzer 2100 (Agilent, Palo Alto, CA). Specific activity measurements of the refolded cutinase-scFv and cutinase- V_{HH} fusion proteins demonstrated that 100% of the cutinase activity was restored, although the degree of refolding for the attached antibody fragments may be less.

Surface Plasmon Resonance (SPR) Spectroscopy. Substrates for SPR spectroscopy were prepared by electron beam evaporation of titanium (40 Å) followed by gold (450 Å) onto glass coverslips, using a Thermionics e-GUN evaporator. Mixed monolayers were formed by immersing gold-coated glass coverslips (1 cm²) in ethanolic solutions containing an asymmetric phosphonate- and tri(ethylene glycol)-terminated disulfide (0.002–0.04 mM) and the symmetrical tri(ethylene glycol)-terminated disulfide (0.96–0.998 mM) at a total concentration of disulfides of 1 mM for 18 h.¹⁶ After this period, 180 μL of ethanolic tri(ethylene glycol)-terminated alkanethiol was added and kept for 2 h. The substrate was then removed from the solution, washed with deionized water and absolute ethanol, dried with a stream of nitrogen gas, and glued to a SPR sensor chip holder. All SPR spectroscopy experiments were performed with a Biacore 1000 at 25 °C using PBS (pH 7.4) as the running buffer. The cutinase-fused antibodies were applied to the SAMs presenting phosphonate at a flow rate of 1 $\mu\text{L}/\text{min}$ at \sim 300 nM concentration for 40 min, resulting in immobilization. After immobilization of the antibodies, various antigens (10 μM in PBS) were flowed at a rate of 1 $\mu\text{L}/\text{min}$ for 5 min. For the next 5 min, sodium dodecyl sulfate (SDS) in PBS (0.1% w/w) was flowed at a rate of 1 $\mu\text{L}/\text{min}$ to disrupt noncovalent

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protein–protein interactions. In all cases, protein binding was measured in α RU, which is an arbitrary unit used in the Biacore SPR system to represent the extent of resonance angle shift.¹⁹ The absolute amount of bound proteins was determined using the relationship of $1000 \text{ RU} = 0.1^\circ = 1 \text{ ng/mm}^2$.

Fluorescence Assays. Monolayer substrates for fluorescence assays were prepared as described for SPR spectroscopy, using glass coverslips coated with 10 \AA titanium and 100 \AA gold. Fluorescently labeled antigens were prepared using an Alexa Fluor 532 protein labeling kit (Molecular Probes, Eugene, OR). Solutions of each antigen (0.5 mL , 2 mg/mL) in 0.1 M sodium bicarbonate buffer were added to vials of reactive dye. The reaction mixtures were stirred for 1 h at room temperature. Excess dye was removed by dialysis or gel chromatography based on protein size. Solutions of antibody (600 nM in PBS) were spotted and immobilized to SAMs presenting phosphonate capture ligand for 4 h using a poly-(dimethyl siloxane) (PDMS) stencil to prevent spreading of droplets. The PDMS (Sylgard 184, Dow Corning) stencil was prepared by punching holes using a blunt end needle on a $2.5 \text{ cm} \times 4 \text{ cm} \times 0.5 \text{ cm}$ (length \times width \times height) PDMS membrane prepared according to standard procedures.²⁰ The PDMS stencil was blocked prior to use with PBS containing 1% BSA and 0.04% Tween 20 (24 h , 4°C). The protein solutions were removed, and each well was washed twice with $10\text{-}\mu\text{L}$ fractions of both PBS and PBST (PBS containing 0.04% Tween 20). In each well, solutions of fluorescently labeled antigens ($5 \mu\text{L}$, $20 \mu\text{M}$) were added, incubated for 2 h at room temperature, and then washed. The PDMS stencil was removed, and the gold substrate was rinsed with PBS, PBST, and distilled water, and dried with a stream of nitrogen gas. The array was scanned in a GenePix 4000B microarray fluorescence scanner using a 532-nm wavelength laser source.

RESULTS

Cloning, Expression, and Purification of Cutinase-Fused Antibodies. A plasmid was constructed to express cutinase fusions of antibodies (Figure 1B). In this vector, a $(\text{GGGG})_3$ flexible linker was incorporated between genes encoding cutinase and the fusion partner to facilitate independent folding of the fused proteins. Antibodies used for the fusion construction were two scFvs that recognize the SH3 domain of human c-Src and the N-terminal EH domain of frog intersectin, one V_{HH} that recognizes hen lysozyme, and two FN3 monobodies that recognize the Src SH3 domain and streptavidin. The cutinase and cutinase-FN3 fusions were expressed in a soluble, functional form, whereas the V_{HH} and scFv fusions accumulated as insoluble aggregates. For the latter proteins, urea (8 M) was used to denature the aggregates, and a reducing environment was generated to promote formation of disulfide bonds in the antibody segments of the fusion proteins. A dissociation constant of 10 nM was measured by isothermal titration calorimetry for the interaction of purified cutinase-fused V_{HH} with lysozyme, in excellent agreement with the value (13 nM) reported for the nonfused form of the antibody.¹¹ Each cutinase fusion was purified to homogeneity, and

the molecular weight of each fusion agreed with expected values (Figure 1C).

Preparation of Self-Assembled Monolayers for Antibody Immobilization. SAMs were prepared from phosphonate- and tri(ethylene glycol)-terminated disulfides as previously described.¹⁶ Briefly, gold-coated glass coverslips were immersed in ethanolic solutions of a phosphonate- and tri(ethylene glycol)-terminated disulfide and a symmetrical tri(ethylene glycol)-terminated disulfide (in a ratio of 1:99) for 18 h at a total disulfide concentration of 1 mM . Next, an ethanolic solution of tri(ethylene glycol)-terminated alkanethiol was added, and the monolayer was further incubated for 2 h . The density of the phosphonate capture ligand was adjusted by varying the ratio of the two disulfides in the initial monolayer formation. Cutinase fusion proteins were captured by the ligand on SAMs as diagrammed in Figure 2. The tri(ethylene glycol) groups served to prevent nonspecific adsorption of proteins to the monolayer.²¹

Immobilization of Cutinase-Anti-Lysozyme V_{HH} . We used SPR spectroscopy to characterize the immobilization of cutinase-anti-lysozyme V_{HH} (cuti- V_{HH} /lysozyme) to SAMs presenting the phosphonate capture ligand. In these experiments, PBS ($\text{pH } 7.4$) was flowed over the monolayer for 3 min to establish a baseline, followed by a solution of cuti- V_{HH} /lysozyme (300 nM) in the same buffer for 40 min to observe binding. The protein solution was replaced with the buffer for 5 min to measure the amount of protein that remained immobilized (Figure 3A). The binding of cuti- V_{HH} /lysozyme to the substrate was irreversible and not affected by 0.1% sodium dodecyl sulfate (SDS) in PBS (data not shown). SDS is a detergent that denatures proteins and serves to remove noncovalently adsorbed proteins from surfaces.²² Control experiments verified that the immobilization of cuti- V_{HH} /lysozyme was blocked when the protein was first incubated with a soluble phosphonate ligand, demonstrating that the immobilization is specific (data not shown). On the basis of the structure of cutinase,²³ we introduced a mutation (H188R) in the active site triad (Ser120, Asp175, and His188) of cutinase, rendering the enzyme inactive. The mutated cuti- V_{HH} /lysozyme bound to the substrate, but did not result in covalent immobilization, as evidenced by its removal when treated with SDS (data not shown). This result further supports the importance of a covalent adduct between the cutinase and the phosphate ligand for stable immobilization of antibodies.

The activity of immobilized cuti- V_{HH} /lysozyme was measured by flowing a solution of lysozyme ($10 \mu\text{M}$ in PBS) over the immobilized cuti- V_{HH} /lysozyme for 5 min (Figure 3A). The lysozyme solution was replaced with the buffer to measure the amount of lysozyme that remained bound to the immobilized cuti- V_{HH} /lysozyme. The amount of cuti- V_{HH} /lysozyme that was immobilized and the amount of lysozyme that bound to the antibody were determined as described in the experimental section. The absolute amount of immobilized cuti- V_{HH} /lysozyme ($\text{MW} = 39$

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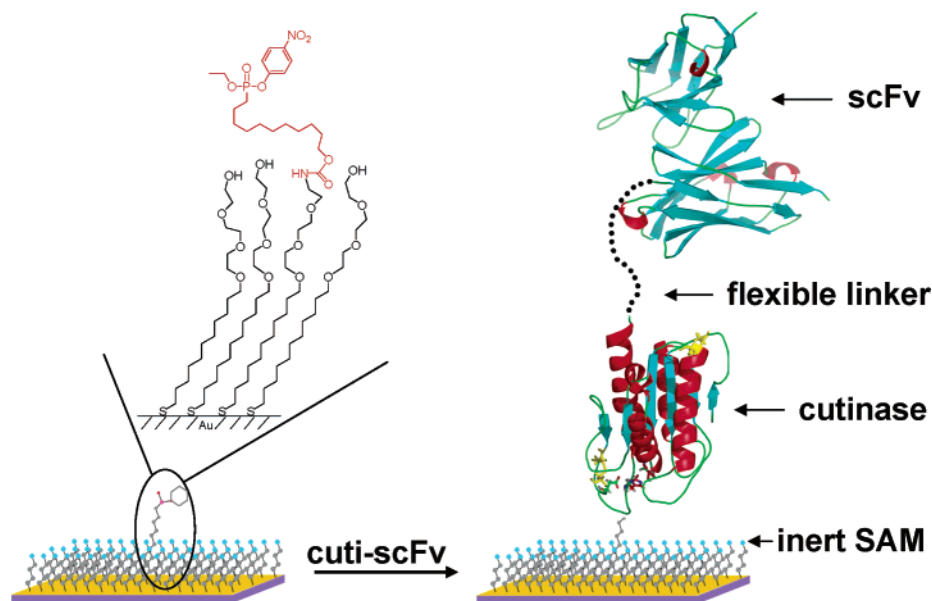


Figure 2. The structure of a self-assembled monolayer (SAM) presenting a capture ligand and immobilization of a cutinase-fused antibody. The monolayer and protein are drawn to scale. The PyMOL Molecular Graphics system (DeLano Scientific, San Carlos, CA) was used to generate the figure of cutinase and scFv molecules; cyan, red, and green regions correspond to α -sheet, α -helical, and loop structures, respectively. Phosphonate capture ligand was present at a density of approximately 0.5% relative to total alkanethiolate. The tri(ethylene glycol) group prevents nonspecific adsorption of proteins to SAMs. The cuti-scFv fusion is covalently immobilized to the phosphonate capture ligand by way of the cutinase domain, distancing the antigen-binding site of the scFv away from the solid surface, through a flexible linker.

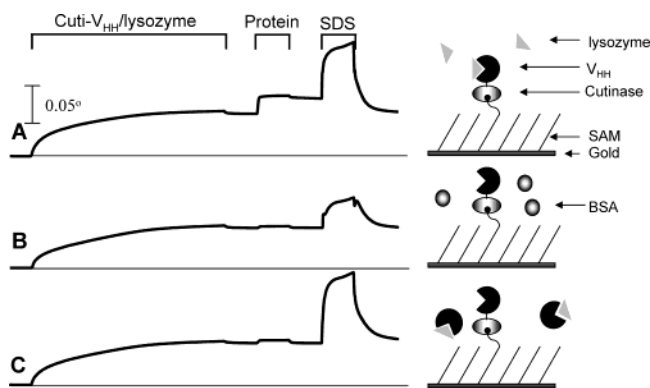


Figure 3. SPR spectroscopy data of the cutinase-fused V_{HH} /lysozyme immobilization. (A) Cuti- V_{HH} /lysozyme was immobilized to an SPR sensor chip presenting a phosphonate capture ligand. Immobilized V_{HH} /lysozyme retained its binding activity for lysozyme, and SDS treatment selectively destroyed the noncovalent protein–protein interaction. (B) BSA did not bind to the immobilized V_{HH} /lysozyme. (C) Preincubation of lysozyme with soluble cuti- V_{HH} /lysozyme-blocked binding of lysozyme to the immobilized V_{HH} /lysozyme.

kDa) was determined to be $15.1 \text{ fmol}/\text{mm}^2$, and the amount of bound lysozyme (MW = 14.3 kDa) was $14.4 \text{ fmol}/\text{mm}^2$. These findings indicate that 95% of the immobilized cuti- V_{HH} /lysozyme was functionally active. In the control experiment, BSA did not show significant binding to cuti- V_{HH} /lysozyme ($\alpha\text{RU} < 15$), demonstrating that the tri(ethylene glycol)-terminated SAM was otherwise inert to protein adsorption (Figure 3B). As expected, the binding of lysozyme to immobilized V_{HH} was prevented when the lysozyme was incubated with soluble cuti- V_{HH} /lysozyme prior to the binding experiment (Figure 3C).

Immobilization and Binding of Cutinase-Fused Antibodies. We next demonstrated the immobilization of four additional fusion proteins: two scFvs and two FN3 monobodies. These

experiments demonstrate that the cutinase-mediated immobilization strategy can be applied to the immobilization of several affinity reagents. SPR was used to monitor the immobilization of the following fusion proteins: cutinase-anti-Src SH3 scFv (cuti-scFv/Src SH3), cutinase-anti-EH scFv (cuti-scFv/EH), cutinase-anti-Src SH3 FN3 (cuti-FN3/Src SH3), and cutinase-anti-streptavidin FN3 (cuti-FN3/streptavidin) (Figure 4). In each case, the cutinase fusions were covalently immobilized to the monolayers presenting the capture ligand. Each of the immobilized antibodies bound their respective antigens and released the antigen upon exposure to 0.1% SDS.

We used SPR to determine the amount of antigen that bound each antibody under saturating conditions, as described above for the cuti- V_{HH} /lysozyme fusion protein. We again compared the amount of antigen that bound to the amount of antibody that was immobilized. On the basis of these measurements, and with the assumption of a 1:1 complex of antigen and antibody, we found that the following fractions of immobilized antibody were capable of binding antigens: 73% for cuti-scFv/Src SH3, 95% for cuti- V_{HH} /lysozyme, 84% for cuti-FN3/Src SH3, and 21% for cuti-FN3/streptavidin. The immobilized antibodies all showed high activities with the exception of cuti-FN3/streptavidin. We also note that the binding of GST-EH to cuti-scFv/GST-EH occurred with a stoichiometry that was greater than 1:1; we interpret the excess binding of antigen to be due to the dimerization of the GST fusion protein.²⁴

To compare the cross-reactivity between the antigens and the immobilized antibodies, SPR spectroscopy was used to measure the binding of each antigen to each immobilized antibody (Figure 5A). The experiments, described previously, were repeated with new substrates for each experiment (i.e., the immobilized antibodies were not used for multiple binding experiments). With the

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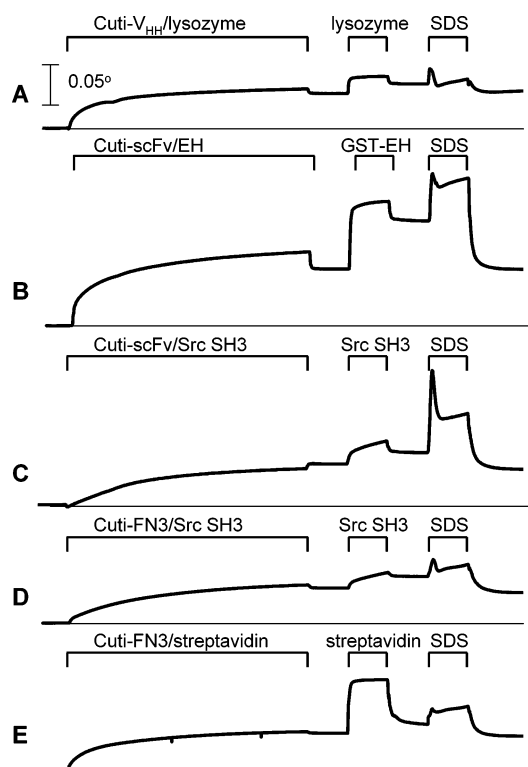


Figure 4. SPR data for five immobilized cutinase fusion proteins. The examples include antibodies derived from V_{HH} , scFv, and FN3 domains. Antibodies were immobilized to SAMs presenting the capture ligand and the binding activity for corresponding antigens was monitored as shown in Figure 3. Data correspond to the following antibodies and antigens: (A) cuti- V_{HH} /lysozyme and lysozyme, (B) cuti-scFv/EH and EH, (C) cuti-scFv/Src SH3 and Src SH3, (D) cuti-FN3/SH3 and SrcSH3, and (E) cuti-FN3/streptavidin and streptavidin.

exception of GST–EH binding to the immobilized cuti-scFv/Src SH3, each of the antibodies showed a strong selectivity for the target antigens. In the case of the former, we interpret the cross-reactivity to be due to the use of an antigen constructed as a GST fusion, which resulted in selection of an engineered scFv that binds to both GST and Src SH3 portions of the fusion. In support of this interpretation, some binding of free GST to the immobilized cuti-scFv/Src SH3 was in fact detected (data not shown).

We next showed that the antibody–antigen interaction could be detected in an array format using fluorescence imaging to identify binding of labeled antigens (Figure 5B). Arrays of antibodies were generated by immobilizing cutinase (as a negative control), cuti-scFv/EH, cuti-scFv/Src SH3, cuti- V_{HH} /lysozyme, cuti-FN3/Src SH3, and cuti-FN3/streptavidin in several spots in each of six columns. To prevent uncontrolled spreading of the cutinase fusions during the immobilization, we first applied an elastomeric stencil to the monolayer. The stencil contained an array of 30 holes which defined the regions on the substrate to which the antibodies immobilized. The stencil was blocked with BSA prior to use to minimize protein adsorption to the hydrophobic material. After immobilization, the wells were rinsed and the fluorescently labeled antigens were added across the rows, as illustrated in Figure 5B. Following incubation, the stencil was removed and the substrate was rinsed with buffer. Fluorescence imaging of the substrate revealed specific binding between the antigens and their cognate antibodies, with the exception of GST–EH antigen, as noted before in the SPR experiments.

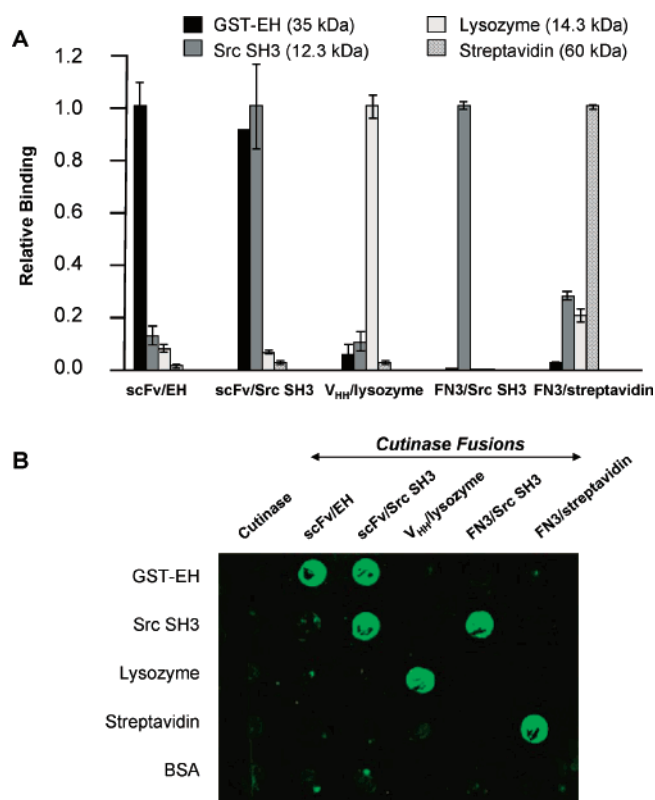


Figure 5. SPR spectroscopy and fluorescence assays demonstrate selectivity. (A) The cross-reactivity for five immobilized antibodies was measured by SPR spectroscopy. All of the immobilized antibodies showed high selectivity for their cognate antigens, except cuti-scFv/Src SH3 (see text for details). Binding activities for each antibody were determined by measuring the moles of antigen that bound per mole of antibody and are reported relative to the specific interaction. (B) The array of antibodies was generated to present cutinase (negative control), cuti-scFv/EH, cuti-scFv/Src SH3, cuti- V_{HH} /lysozyme, cuti-FN3/Src SH3, and cuti-FN3/streptavidin in columns 1–6, respectively. Fluorescently labeled antigens were spotted onto the immobilized antibodies. Binding was only observed between the fluorescently labeled proteins and their corresponding antibodies, except for cuti-scFv/Src SH3, as previously noted in Figure 4. For the SPR spectroscopy experiments, 0.5% phosphonate-terminated alkanethiolate SAMs, 300 nM antibody solution, and 10 μ M antigen solution were used, and for the fluorescence assays 2% phosphonate-terminated alkanethiolate SAMs, 600 nM antibody solution, and 20 μ M antigen solution were used.

Density and Binding Efficiency of Immobilized Antibody.

To illustrate the control that the cutinase-directed immobilization method provides over antibody density, we prepared SAMs that present the phosphonate capture ligand at a range of densities. SAMs were formed from mixtures of the two dialkyl disulfides, with the fraction of chains presenting the phosphonate capture ligand ranging from 0.1% to 2%. Figure 6A shows that the amount of immobilized antibody increased linearly with the density of the capture ligand in the monolayer.²⁵

We next investigated whether the relative activity of the immobilized antibody (defined as the fraction that can bind antigen) depends on the density at which the antibody is

(25) In general, the ratio of two alkanethiolates in a monolayer is not linearly correlated with the ratio of the reagents used during preparation of the monolayers. Our observation of a linear correlation likely reflects the near-linearity of this relationship over a small range of density.

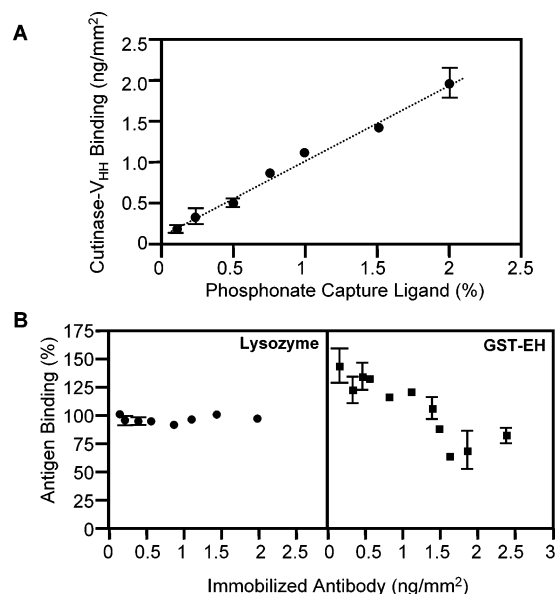


Figure 6. Density control and binding efficiency measurements of the immobilized antibodies. (A) Density of immobilized cuti-V_{HH}/lysozyme was controlled using SAMs presenting phosphonate ligands at different concentrations. The amount of immobilized protein was measured quantitatively using SPR spectroscopy and plotted vs percentage of phosphonate capture ligand in solution. This graph shows that the amount of protein immobilized increased proportionally as the solution concentration of phosphonate capture ligand increased. (B) Binding efficiency and crowding effect of antigen binding to immobilized antibodies are studied. Soluble lysozyme shows 90–100% binding to immobilized cuti-V_{HH} (39 kDa)/lysozyme (14.3 kDa) in densities of 0.3–2 ng/mm². When a relatively larger antibody–antigen pair, cuti-scFv (52 kDa)/GST–EH (35 kDa), was used, we observe that the binding efficiency decreases from 150% to 60% as the density of immobilized scFv/EH increases from 0.3 ng/mm² to 2.5 ng/mm². Error bars indicate standard deviations.

immobilized (Figure 6B). In the first example, we prepared monolayers having the cuti-V_{HH}/lysozyme antibody immobilized at densities ranging from 0.3 to 2 ng/mm². We measured the amount of lysozyme that bound to the immobilized antibodies and found that greater than 90% of the antibody was active across this series of monolayers. This uniform level of activity agrees with the relative sizes of the immobilized antibody and antigen. Lysozyme is smaller than the antibody, and therefore, binding of lysozyme to an antibody does not preclude binding of a second lysozyme to a neighboring antibody. In the second example, we immobilized an antibody for the large antigen GST–EH (35 kDa). In this example, we found that the fraction of the immobilized antibody that was active for binding antigen decreased from 150% to 60% as the density of immobilized antibody was increased from 0.3 ng/mm² to 2.5 ng/mm². As noted earlier, these fractional activities are based on a 1:1 stoichiometry of antigen and antibody, but in this case dimerization of two GST domains can lead to a greater amount of antigen binding the substrate. In any event, the data clearly show that as the density of antibody is increased, steric crowding of the antigen–antibody complexes lowers the activity of the immobilized antibody. This experiment clearly illustrates that the amount of antigen that binds to an array depends on the density of the immobilized antibody.

Detection of Antigen in a Complex Solution. The results thus far have demonstrated the use of immobilized antibodies for

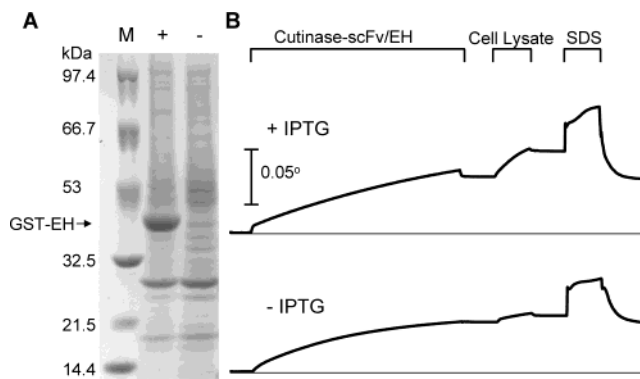


Figure 7. Detection of antigen in a bacterial cell lysate. (A) Cell lysates from *E. coli* transformed with EH-expressing plasmid were analyzed by SDS–PAGE: (+) cell lysate from IPTG-treated cells, (–) cell lysate from cells without IPTG treatment. The total amount of protein in both samples was the same. The mobility of GST–EH (arrow) is compared to that of reference proteins (lane M). (B) Detection of EH in a complex solution was accomplished using SPR spectroscopy. Significant binding was observed only when the sample contained overexpressed EH.

recognition of antigens in simple solutions. In practice, it is important for antibody chips to be capable of detecting antigens in complex solutions (e.g., cell lysate, blood, urine). To examine the ability of our substrate to function in complex solutions, we prepared two cell lysates from bacterial cells, both of which carried the GST–EH expression plasmid. One lysate was prepared from cells that were induced with IPTG to overexpress GST–EH ((+) cell lysate) and the other lysate was prepared from cells without IPTG treatment ((–) cell lysate), leading to no, or only leaky levels of, GST–EH expression. The levels of GST–EH protein was analyzed with a 4–20% Tris–glycine gradient under denaturing conditions (Figure 7A) to confirm expression in induced cells. When the two cell lysates were incubated with a monolayer displaying the cuti-scFv/EH fusion, only the induced cell lysate demonstrated the presence of antigen (Figure 7B). This result shows that this system is compatible with detection of specific proteins in complex biological samples and is, therefore, relevant to the range of applications for antibody arrays.

DISCUSSION

In this paper, we immobilized five fusion proteins containing antibodies of three different types (scFv, V_{HH}, and FN3) to the SAMs presenting a phosphonate capture ligand. This immobilization was completed in a short period of time—within 5 min with antibody solutions at 10 μM concentration. The rapid rate of antibody immobilization will minimize the amount of antibody needed in preparation of substrates and reduce complications arising from solvent evaporation. An important aspect of this work is the demonstration that a high fraction of the immobilized antibodies retained activity for recognition of their cognate antigens.²⁹ In contrast, the antibody arrays that were prepared through direct adsorption of antibodies to glass substrates coated with poly-L-lysine showed that approximately 20% of immobilized

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antibodies retained their specific activity.^{5a} Furthermore, the arrays demonstrated here produced very low background signals and did without employing blocking procedures on the substrate. Many of the strategies now used to prepare arrays achieve a higher density of immobilized antibody—through the use of gels on the substrate³⁰—and therefore provide a larger binding capacity for antigen. However, it remains to be determined whether the background signals, which can limit the sensitivity and dynamic range in detection assays with complex biological samples, will ultimately favor two- or three-dimensional arrays.

An important challenge in assembling protein chips is the need to present each of the different proteins in a conformation and orientation that ensures biological activity.^{6–8} For antibodies, this challenge translates to maximizing the fraction of immobilized proteins that bind the target antigen. In an ideal case, all of the immobilized antibodies will bind the antigen with an identical affinity. In practice with previous approaches, less than one-half of the immobilized antibodies are functional, and even then, they bind antigen with a range of affinities.⁵ We expect that this fractional activity should be minimized when the antibody is uniformly oriented and immobilized at a density that maximizes the number of bound antigens molecules, without introducing interactions between neighboring antibody–antigen complexes.

This paper describes a method that meets these goals and provides an effective strategy for preparing antibody chips. The cutinase fusion strategy ensures that the immobilized antibodies are uniformly oriented, because the cutinase domain undergoes a site-specific immobilization to the substrate. While conformational flexibility in the (GGGG)₃ spacer between the two domains allows the antibody to assume a distribution of orientations, each antibody is expected to have the same distribution of orientations and, therefore, can assume the same favorable conformation(s) to optimize binding to antigens. Furthermore, this method gives

straightforward control over the density of the immobilized proteins. Since the immobilization of a cutinase fusion protein requires a covalent reaction with the phosphonate ligand of the substrate, the number of proteins that can be immobilized to a defined area of the substrate is controlled by the density of the phosphonate capture ligand. For monolayer substrates, this density is easily controlled by adjusting the ratio of the two disulfides used to prepare the monolayer.²⁶ This approach to density control is an improvement over current strategies, which monitor the rate of immobilization and terminate the reaction when the desired amount of protein has been loaded on the substrate.⁷ These kinetic approaches, which tend to require substantial optimization and can suffer from a lack of reproducibility, are required because the substrates contain many functional groups that can be used for protein immobilization, and it is difficult to control the densities of these functional groups in order to limit protein immobilization.

The use of alkanethiolates SAMs on gold as the substrate for the antibody array is integral to both the preparation and performance of the chip. While monolayers can have defects and other structural irregularities (for example, those that arise because the gold substrates are not atomically flat over large areas), these heterogeneities are modest when compared to polymer substrates. A central feature of the monolayers is the oligo(ethylene glycol) group that serves as the background to the immobilized ligands. Monolayers presenting the glycol groups are among the most effective substrates at preventing the nonspecific adsorption of protein, which is important for the selectivity of the immobilization reaction and elimination of background signals in assays.²² The combination of (1) methods for immobilizing ligands, (2) uniform environments on the surface, and (3) resistance to unwanted protein adsorption has made monolayer substrates well suited for quantitative bioanalytical assays,²⁷ as has been demonstrated with monolayers presenting peptides,^{28a–c} carbohydrates,^{28d,e} and proteins.^{28f–h} In the future, it will be important to devise methods for convenient fabrication of high-content arrays and evaluate their stability under dry conditions (i.e., shelf life). Finally, the use of biochips requires that they be characterized with analytical detection methods. SAMs are compatible with the most widely used methods, including surface enhanced Raman spectroscopy, fluorescence spectroscopy, mass spectroscopy, radioactivity, and two-dimensional SPR spectroscopy.^{28i–l}

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