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Regioselective Covalent Immobilization of Recombinant Antibody-Binding Proteins A, G, and L for Construction of Antibody Arrays

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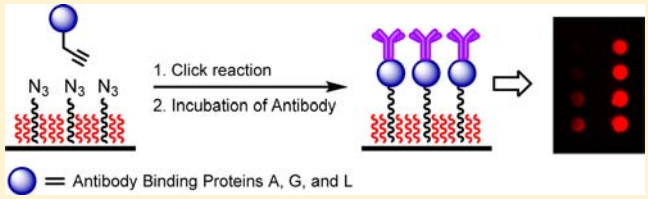
Supporting Information

ABSTRACT: Immobilized antibodies are useful for the detection of antigens in highly sensitive microarray diagnostic applications. Arrays with the antibodies attached regioselectively in a uniform orientation are typically more sensitive than those with random orientations. Direct regioselective immobilization of antibodies on a solid support typically requires a modified form of the protein. We now report a general approach for the regioselective attachment of antibodies to a surface using truncated forms of antibody-binding proteins A, G, and L that retain the structural motifs required for antibody binding. The recombinant proteins have a C-terminal CVIX protein farnesyltransferase recognition motif that allows us to append a bioorthogonal azide or alkyne moiety and use the Cu(I)-catalyzed Huisgen cycloaddition to attach the binding proteins to a suitably modified glass surface. This approach offers several advantages. The recombinant antibody-binding proteins are produced in *Escherichia coli*, chemoselectively modified posttranslationally in the cell-free homogenate, and directly attached to the glass surface without the need for purification at any stage of the process. Complexes between immobilized recombinant proteins A, G, and L and their respective strongly bound antibodies were stable to repeated washing with PBST buffer at pH 7.2. However, the antibodies could be stripped from the slides by treatment with 0.1 M glycine·HCl buffer, pH 2.6, for 30 min and regenerated by shaking with PBS buffer, pH 7.2, at 4 °C overnight. The recombinant forms of proteins A, G, and L can be used separately or in combination to give glass surfaces capable of binding a wide variety of antibodies.



INTRODUCTION

Posttranslational modification of proteins to introduce novel functional groups is an increasingly important strategy for construction of protein biochips used to characterize the interactions or biochemical activities of proteins by high-throughput techniques that can be conducted with small quantities of materials.[1−3] By immobilizing proteins on a planar solid surface, several thousand assays can be conducted on a single chip, in contrast to the substantially lower capacity of standard solution-phase microtiter assays. The advantages of chip technology are evident in the widespread use of DNA microarrays.[4] However, construction of protein microchips can be substantially more difficult than that of their DNA counterparts. Typically, proteins are more sensitive molecules whose native folds must be preserved throughout the immobilization process in order to retain biological function.[5] Ideally, the reactions needed to immobilize proteins should be chemo- and regioselective and proceed under mild conditions in an aqueous buffer while avoiding reagents, pH's, and temperatures that could result in denaturation.[6−8] In addition, the proteins must be compatible with the solid surface of the chip.

While the simplest approach to immobilization involves non-covalent adsorption of the native protein onto a modified surface through polar, ionic, or hydrophobic interactions, the binding site of a randomly immobilized protein may be fully or partially blocked, resulting in a heterogeneous output, false negatives, or decreased signal intensity.[9,10] These problems can be circumvented when the proteins are immobilized regioselectively, either non-covalently through poly-histidine/nickel, biotin/streptavidin, or hydrophobic interactions or covalently.[11,12] Of these approaches, covalent attachment is particularly attractive because the proteins cannot dissociate from the surface during an analysis.

Regioselective immobilization, either covalent or non-covalent, typically involves a regioselective modification of the wild-type protein with a non-native moiety. We have used a regioselective, biocompatible modification−immobilization strategy based on the posttranslational farnesylation of proteins bearing a C-terminal CaaX motif.[13] The CaaX tetrapeptide, where "C" is cysteine, "X" is serine, alanine, methionine, or glutamine, and "a" is an amino acid with a small hydrophobic side chain, is the recognition motif for protein farnesyltransferase (PFTase), which adds the farnesyl moiety in farnesyl diphosphate (FPP) to the cysteine thiol.[14] The modification reaction is general for proteins bearing a CaaX motif, and as a result, it is possible to add a farnesyl unit to virtually any soluble protein by modifying the corresponding gene to append the CaaX unit.

We discovered that simple analogues of FPP where the ω-isoprene unit was replaced by propargyl ether or alkyl azide

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moieties are excellent substrates for PFTase and efficiently afford proteins bearing chemically stable bioorthogonal azide or alkyne functional groups.[15] The modified proteins can then be attached to glass surfaces bearing complementary azide or alkyne moieties by a Cu^{+}-catalyzed Huisgen cycloaddition reaction. As a result of the high selectivity of PFTase-catalyzed posttranslational modification and the Huisgen cycloaddition, the CaaX cysteine of a recombinant protein in the crude supernatant from an *Escherichia coli* expression plasmid can be alkylated and the resulting modified protein immobilized on the surface of a glass slide without purification at any stage of the process under conditions that preserve the native fold and activity of the protein.[13,16]

Highly efficient immobilization of antibodies on solid surfaces is crucial for development of immunobiosensors that can detect a wide variety of analytes, including drugs, toxins, bacteria, pathogens, and metabolic biomarkers.[17−19] Among the many strategies that have been reported is the use of immobilized forms of antibody-binding proteins from *Staphylococcus aureus* (protein A), *Streptococcus C40* (protein G), and *Peptostreptococcus magnus* (protein L) to bind the antibodies to the surface.[20] Proteins A and G bind to the Fc region in the heavy chains, while protein L binds to κ-light chains outside of the antigen-binding site. Structural studies show that well-defined motifs—domains E, D, A, B, and C in protein A; C1, D1, and C2 in protein G; B1, B2, B3, and B4 in protein L—are responsible for binding.[21] Proteins A, G, and L bind a wide variety of antibodies with different affinities (see Table S1) and have been used to immobilize antibodies in protein-based microarrays.[22,23]

The sensitivity of antibody-based assays is increased when the antibodies are immobilized in well-controlled orientations that expose the antigen-binding site with enhancements of as much as 100-fold for antibodies bound to immobilized protein A versus randomly oriented binding.[24] Several strategies have been used to immobilize antibody-binding proteins, including hydrophobic interactions between a glass surface and protein A, G, or L fusion proteins, cysteine thiol−gold interactions, cross-linking through cysteine and lysine residues, and complementary DNA−DNA interactions.[25−28] We now describe procedures for covalently immobilizing truncated versions of proteins A, G, and L, which retain their respective antibody-binding motifs, in a well-defined orientation using a regiospecific chemoenzymatic approach based on bioorthogonal reactions that eliminate the need to purify the recombinant proteins at any stage of the process. Glass slides coated with the recombinant proteins A, G, and L selectively bind their respective antibody targets and can be stripped of the antibodies and reused.

EXPERIMENTAL SECTION

Construction of Expression Plasmids for Antibody-Binding Proteins with C-Terminal CVIA Tags. Regions encoding the antibody-binding sites in proteins A, G, and L in plasmids SpA-pGEX-KG, proLG-pHD389, and proL-pHD389,[29−31] respectively, were subcloned into pET-28b(+) (Novagen), and each was modified to append a C-terminal RTRCVIA farnesyl transferase recognition site to the expressed proteins. A 885 bp region coding the E, D, A, B, and C domains of *S. aureus* protein A was amplified with PfuUltra HF DNA polymerase (Stratagene) using primers containing a 5′ *Nde*I site and a 3′ *Xho*I site (see SI). PCR conditions (30 cycles) were as follow: initial denaturation, 95 °C, 120 s; denaturation, 95 °C, 30 s; annealing, 52.1 °C, 60 s; extension, 72 °C, 60 s; final extension, 72 °C, 10 min. The PCR products were purified on 1% agarose and extracted using the GFX PCR DNA gel purification kit (GE Healthcare). The purified PCR fragments and pET28b(+) were doubly digested with *Nde*I and *Xho*I and ligated using T4 DNA ligase (New England Biolabs) to give plasmid pProA-CVIA. *E. coli* XL10-Gold (Stratagene) was transformed with pProA-CVIA, and individual colonies were picked from LB/Kan (LB with 30 μg/mL kanamycin) plates. Plasmid from the transformants was isolated and sequenced to confirm the structure of the construct. The encoded protein (proA-CVIA) contained five antibody-binding domains (E, D, A, B, and C) from protein A with a His_6-tag and a thrombin proteolytic site at the N-terminus and a RTRCVIA farnesyl recognition site at the C-terminus. A four amino acid EEDN sequence was inserted between the C-domain and RTRCVIA. PCR was not successful without the EEDN insert due to the high homology between domains E, A, D, and B and domain C.

Similar expression plasmids for CVIA-tagged protein G (proG-CVIA) and protein L (proL-CVIA) were constructed using same procedure described for proA-CVIA with minor modifications. A 375 bp sequence encoding the C1, D1, and C2 domains of *Streptococcus* C40 protein G was amplified with PfuUltra HF DNA polymerase (Stratagene) using primers containing a 5′ *Nde*I site and a 3′ *Xho*I site (see SI) and ProLG-pHD389 as a PCR template. The B1, B2, B3, and B4 domains of *Peptostreptococcus magnus* protein L were amplified with PfuUltra HF DNA polymerase (Stratagene) using primers containing a 5′ *Nde*I site and a 3′ *Xho*I site (see SI) and ProLG-pHD389 as a PCR template. The PCR conditions were identical with those used for pProA-CVIA except for the annealing steps: 43.7 °C for the protein G plasmid and 47.1 °C for the protein L plasmid, respectively. Procedures for restriction digestion and ligation of plasmids were same as those for pProA-CVIA. The final expression plasmids pProG-CVIA and pProL-CVIA encoded proG-CVIA and proL-CVIA, respectively. pProG-CVIA has a single silent G816A mutation. pProL-CVIA has a single A340G mutation, which corresponds to a T114A mutation in the protein. Attempts to remove the mutation by site-directed mutagenesis and fusion cloning failed, probably due to the high homology among the B1−B4 domains. Western blots of purified proL-CVIA with monoclonal anti-GFP mouse antibody showed strong binding. The ambidextrous Ig binding of protein L renders it relatively robust to mutations.[32,33]

Expression and Purification of ProA-CVIA, ProG-CVIA, and ProL-CVIA. Plasmids pProA-CVIA and pProG-CVIA were transformed separately into BL21(DE3). Starter cultures (5 mL of LB/Kan) of the transformants from an incubation at 250 rpm at 37 °C for 10 h were used to inoculate 100 mL of LB/Kan and grown overnight at 37 °C. A 50 mL portion of the culture was used to inoculate 1.5 L of LB/Kan and was incubated at 37 °C with shaking at 250 rpm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to each culture to a final concentration of 1 mM at $OD_{600} \approx 0.6$, and incubation was continued for 6 h at 37 °C.

Cells were harvested by centrifugation for 20 min at 4 °C at 5000*g*. Cell paste (4 g) was suspended in 12 mL of lysis buffer consisting of 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole, DNaseI, and a protease inhibitor cocktail tablet (Roche). The resuspended cells were lysed by incubation with 1 mg/mL lysozyme for 1 h on ice followed by sonication (5 sets of 30 pulses), and the resulting homogenate was centrifuged at 10000*g* for 25 min to remove cellular debris. A 5 mL portion of Ni-nitrilotriacetic acid (NTA) agarose resin (Qiagen) was added to the resulting supernatant, and the suspension was incubated at 4 °C for 1 h with shaking at 100 rpm. The slurry was loaded onto Ni-NTA column and the flow through was collected. The column was washed with 20 mL of 10 mM imidazole, 300 mM NaCl in 50 mM sodium phosphate buffer, pH 8.0, followed by 30 mL of 20 mM imidazole, 300 mM NaCl, 50 mM sodium phosphate buffer, pH 8.0. The proteins were then eluted with 12 mL of 250 mM imidazole, 300 mM NaCl in 50 mM sodium phosphate buffer, pH 8.0; concentrated using ultrafiltration (Centriprep, YM-10, Millipore), and dialyzed against 20% (v/v) glycerol, 50 mM HEPES (pH 7.5) containing 0.1 mM DTT. Approximately 30 mg each of proteins A-CVIA and G-CVIA were obtained after dialysis. Both proteins were >95% pure based on SDS-PAGE. The proteins were flash-frozen and stored at −80 °C until needed.

For preparation of proL-CVIA, plasmid pProL-CVIA was transformed into Rosetta (DE3). The transformant was cultivated in LB supplemented with 30 μL/mL kanamycin and 34 μg/mL chloramphenicol. The procedures for expression and purification were same with those for proA-CVIA and proG-CVIA. Approximately 40 mg of proL-CVIA, >95% pure based on SDS-PAGE, was obtained. The protein was flash-frozen and stored at −80 °C until needed.

Posttranslational Modification of ProA-CVIA, ProG-CVIA, and ProL-CVIA. ProA-CVIA, proG-CVIA, or proL-CVIA (20 μL, 350 μM) and propargyl farnesyl diphosphate (PFPP)[15] (6 μL, 1.8 mM) were added to 156 μL of 25 mM phosphate buffer, pH 7.0, containing 10 mM $MgCl_2$ and 10 μM $ZnSO_4$. The samples were incubated at 30 °C for 10 min before addition of 10 μL of yeast PFTase (250 nM). After 1.5 h at 30 °C, an additional 10 μL of PFTase was added, and the incubation was continued for 1.5 h. The samples were concentrated using a Centricon YM-10 instrument (Millipore), and excess PFPP was removed on a NAP-5 column. Protein concentrations were determined by Bradford analysis.[34] Alternatively, posttranslational modifications were carried out using clarified cell-free homogenates. The samples were stored at −20 °C.

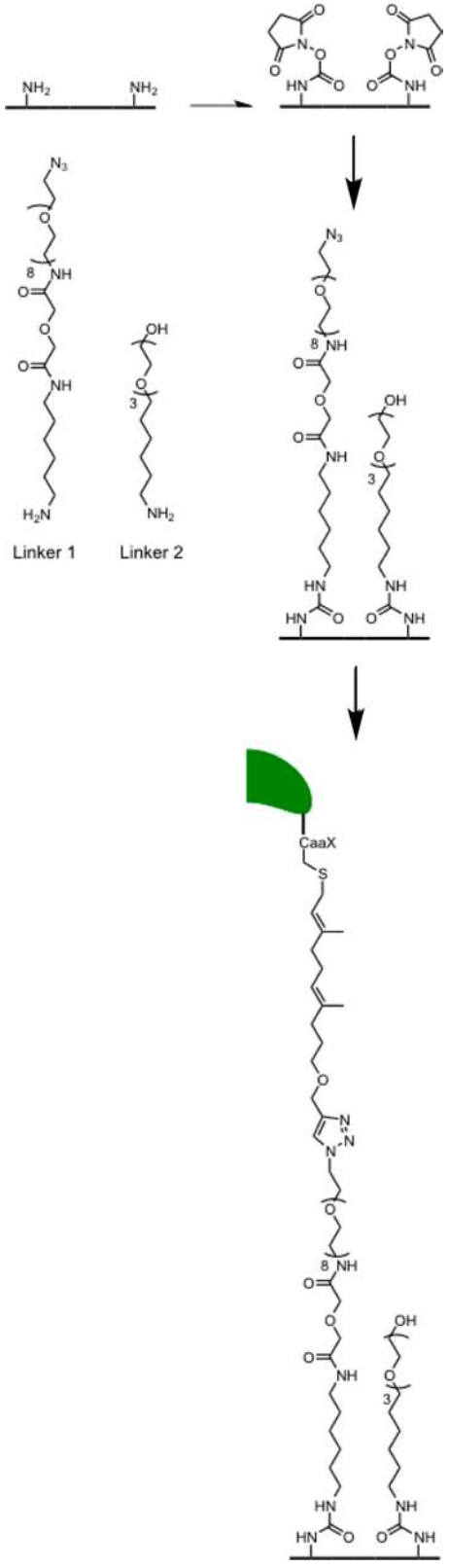
Positive ion electrospray mass spectra of proA-CVIA, proG-CVIA, and proL-CVIA gave the following molecular ions: proA-CVIA, *m/z* calculated for 36 268.7, found 36 268.7; proG-CVIA, *m/z* calculated for 16 611.3, found 16 610.9; proL-CVIA, *m/z* calculated for 35 229.9, found 35 227.9. Positive ion spectra of the modified proteins gave the following molecular ions: proA-CVIApf, *m/z* calculated for 36 487.0, found 36 484.9; proG-CVIApf, *m/z* calculated for 16 829.7, found 16 829.1; proL-CVIApf, *m/z* calculated for 35 448.2, found 35 445.6. None of the proteins had an N-terminal Met, which was presumably removed by methionyl aminopeptidase in *E. coli*.[35] The mass spectra also showed a small peak at M+178 Da, consistent with α-*N*-gluconoylation of the His-tag.[36]

Preparation of Azido-Modified Glass Slides.[13] *N,N′*-Disuccinimidyl carbonate (DSC, 25.8 mg, 0.101 mmol) was dissolved in 2.7 mL of *N,N*-dimethylformamide (DMF), and 0.303 mL of *N,N*-diisopropylethylamine (DIPEA, 1.74 mmol) was added. The DSC−DIPEA solution (3 mL/slide) was added to a reaction chamber. The chamber was purged with N_2, sealed, and shaken at rt overnight at 60−90 rpm. The DSC−DIPEA solution was removed, and the glass slide was washed three times with 100 mL of DMF at rt for 20 min. The slide was allowed to dry under N_2 and treated with a mixture of a 1:9 solution of azido linker (Linker 1):unreactive linker (Linker 2) (31 μL of 11 mM Linker 1 and 146 μL of 21 mM Linker 2) and 27.9 μL of DIPEA in 3 mL of DMF (see Scheme 1). The slide was shaken overnight at 60−90 rpm, washed three times with 100 mL of DMF at rt for 20 min as described above, shaken at rt overnight at 60−90 rpm with 40 μL of ethanolamine in 3 mL of DMF, and washed three more times with 100 mL portions of DMF at rt for 20 min.

Preparation of Coated Slides. Buffer for the Huisgen cycloaddition was prepared immediately before use by mixing 20 μL of $CuSO_4$ (100 mM, 16 mg/mL in deionized water), 20 μL of tris(carboxyethyl)phosphine (TCEP, 100 mM, 14.3 mg/0.5 mL in deionized water), 100 μL of tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)-methyl]amine (TBTA, 10 mM, 5.3 mg/mL of 4:1 t-BuOH:DMSO), and 1.8 mL of phosphate-buffered saline (PBS, pH 7.4).

Silicone-Matted Glass Slides with Individual Wells Coated with Antibody-Binding Proteins. An azido-modified glass slide was swirled in 40 mL of PBS, washed in 40 mL of deionized water for 20 min at 4 °C, and dried under N_2. A silicone mat (Sigma, S3810) was attached, and 3 μL of a solution of proA-CVIApf, proG-CVIApf, or proL-CVIApf in PBS buffer followed by 7 μL of the Cu(I) buffer were added. The final concentrations of antibody-binding proteins were typically between 0.1 and 3.2 μM. The slide was placed in a humid chamber and shaken (60−90 rpm) at rt for 4 h. The silicone mat was carefully removed. The slide was washed with twice with PBST (PBS buffer containing 0.1% Tween 20, pH 7.2) at rt in a high-throughput wash station. Three milliliters of blocking solution (1% bovine serum albumin (BSA) in PBST) was added, and the slides were allowed to stand for 2 h at rt or overnight at 4 °C. The slides were washed with PBST at rt in the wash station.

Scheme 1. Preparation of Protein-Coated Glass Slides



Slides Surface-Coated with proA, proG, or proL. An azido-modified glass slide was prepared for coating as described above. The slide was covered with an mSeries LifterSlip coverslip, and 55 μL of a solution prepared by mixing 30 μL of 333 μM proA-CVIApf, proG-CVIApf, or proL-CVIApf with 70 μL of "Huisgen buffer" was inserted at the edge of the coverslip. The slide was shaken at 60−90 rpm for 4

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h at rt. The coverslip was carefully removed, and the slide was washed twice with PBST in the wash station before 3 mL of BSA blocking solution was added to the chamber. The slide was allowed to stand for 2 h at rt or overnight at 4 °C, washed with PBST in the wash station, and dried under N_2 before a silicone mat was attached.

Visualization with Fluorescent Antibodies. The fluorescent antibody was diluted to the appropriate concentration with PBS immediately before use, a 55 μL portion was added to the surface using the coverslip method, and the slides were incubated at rt for 2 h or at 4 °C overnight with shaking at 60−90 rpm. The slides were then washed twice in the wash station at rt with PBST and dried under N_2 before imaging.

Regeneration of Coated Slides. Seven-microliter samples of rabbit anti-goat IgG (5, 10, and 50 μg/mL) were spotted into the wells of a silicone-matted proA-CVIApf surface-coated slide. After incubation overnight at 4 °C, the mat was removed and the slide was washed twice with PBST at rt. The slide was incubated with Alexa 488-labeled goat anti-human IgG (1 μg/mL) overnight at 4 °C. Fluorescence intensities were measured at 532 nm. The slide was treated with stripping buffer (0.1 M glycine·HCl, pH 2.6) for 30 min, shaken with PBS at 4 °C overnight, and scanned at 532 nm. The mat was reapplied, and rabbit anti-goat IgG was again spotted into the wells. The slide was processed as described for the first cycle and visualized again with Alexa 488-labeled goat anti-human IgG.

RESULTS AND DISCUSSION

Cloning, Purification, and in Vitro Modification of Proteins A, G, and L. Although antibody-binding proteins A, G, and L apparently evolved independently, each of their binding motifs consists of multiple domains of 50−60 amino acid repeat sequences. Protein A contains five homologous Ig-binding domains designated E, D, A, B, and C, which bind Fc and Fab. The Fc site is localized to the elbow region at the CH2−CH3 interface, while the Fab site is in the V_H region and apparently does not interfere with antigen binding.[29] Protein G has three binding domains, C1, D1, and C2, which also bind at the CH2−CH3 interface of Fc, although the specific interactions are different from those for protein A binding.[30] Protein L has five homologous domains, B1−B5, that bind to the Fab region, specifically the V_L region of kappa light chains found in approximately two-thirds of mammalian antibodies, at a site that does not interfere with antigen binding.[31,33]

We constructed truncated recombinant forms of proteins A, G, and L that are substantially smaller than the wild-type proteins and retain their ability to bind antibodies, by removing N- and C-terminal sequences that lie outside of the binding domains. For example, protein A with a mass of 51.5 kDa[37] was truncated to a 36 kDa protein containing antibody-binding domains E, D, A, B, and C. In a similar manner, truncated antibody-binding proteins were constructed from domains C1, D1, and C2 (16.6 kDa) in protein G (51.9 kDa) and domains B1−B4 (35.2 kDa) in protein L (109 kDa).[29−31]

In the case of Protein A, the construct included an N-terminal His_6-tag to facilitate purification, the five Ab-binding domains (E, D, A, B, and C) in the wild-type *S. aureus* protein, and a C-terminal EEDNRTRCVIA sequence that included the recognition site for PFTase. Initially, we encountered difficulties in PCR-based cloning, presumably because of the high homology of the individual binding domains. This problem was eliminated when we inserted the four amino acid EEDN sequence between the Ab-binding region and the C-terminal RTRCVIA motif. The resulting construct was inserted into pET28b(+) to give expression plasmid pProA-CVIA. The related expression plasmids for protein G (pProG-CVIA) and protein L (pProL-CVIA) contain the Ab-binding domains C1, D1, and C2 and B1, B2, B3, and B4, respectively, and the CVIA PFTase recognition motif. The PCR cloning introduced a single silent mutation, G816A, into the coding region for protein ProG-CVIA and a single A340G mutation into the coding region for ProL-CVIA corresponding to T114A mutation in the protein. Attempts to restore the wild-type sequence in the antibody-binding motif for ProL-CVIA by site-directed mutagenesis were unsuccessful, presumably because of the high homology between domains B1−B4.

IPTG-induced expression in pProA-CVIA, pProG-CVIA, and pProL-CVIA gave high levels of proteins A-CVIA (proA-CVIA), G-CVIA (proG-CVIA), and L-CVIA (proL-CVIA) based on SDS-PAGE of cell-free extracts. The proteins were purified by Ni affinity chromatography and analyzed by electrospray mass spectrometry (ESI-MS). The recombinant proteins did not have an N-terminal Met, which was presumably removed by methionyl aminopeptidase in the *E. coli* expression strain.[35] In addition, the mass spectra had a small peak at M+178 Da, consistent with α-N-gluconoylation of the His-tag.[36] The cysteine residue in the C-terminal CaaX sequence of proA-CVIA, proG-CVIA, and proL-CVIA was alkylated with PFPP, an analogue of FPP where the ω-isoprene unit was replaced with a propargyl ether to give proA-CVIApf, proG-CVIApf, and proL-CVIApf, respectively, as confirmed by ESI-MS. Western blots showed that a monoclonal anti-GFP mouse antibody bound strongly to the unmodified and modified versions of the antibody-binding proteins (see Figure S1 for proG-CVIA and proL-CVIA). The single T114A mutation in proL-CVIA does not substantially interfere with Ab-binding, as expected based on previous studies.[32,33] In contrast, proA-CVIA and proA-CVIApf bound only weakly to the mouse antibody, as expected based on the binding properties of wild-type protein A.[38]

Binding Studies with ProA-CVIApf, ProG-CVIApf, and ProL-CVIApf: Preparation of Slides with Immobilized Antibody-Binding Proteins. Glass slides were coated with the antibody-binding proteins as outlined in Scheme 1. Amino-derivatized glass slides were activated with disuccinimidyl carbonate and then treated with a mixture of PEG linkers bearing a terminal azide moiety (Linker 1) and a hydroxyl group (Linker 2). ProA-CVIApf, proG-CVIApf, and proL-CVIApf were covalently attached to the azide linker by the Cu^+-catalyzed Huisgen cycloaddition.[39] Control samples were treated in the same manner, except Cu^+ was not included in the cycloaddition buffer.

Selective Binding by Recombinant ProA, ProG, and ProL. ProA-CVIApf, G-CVIApf, and L-CVIApf were spotted in individual wells of separate matted slides and immobilized. Control wells were treated identically except no Cu(I) was added to the incubation buffer. The mat was removed and the slides were washed before treatment with fluorescent antibodies (Figure 1). A concentration-dependent increase in fluorescence intensities was seen for the wells treated with 0.1−0.8 μM solutions of the antibody-binding proteins, while the intensities were similar for the wells treated with 0.8−3.2 μM solutions, indicating that ∼1 μM concentrations of the alkyne-modified proteins were sufficient to saturate the azide sites on the slides. No fluorescence was seen in the control wells, indicating that nonspecific adsorption of the antibody-binding proteins was minimal.

Proteins A and G bind to antibodies at the CH2−CH3 interface of Fc at nearby, but not identical sites. As a result, the strengths of the antibody-binding protein interactions are not

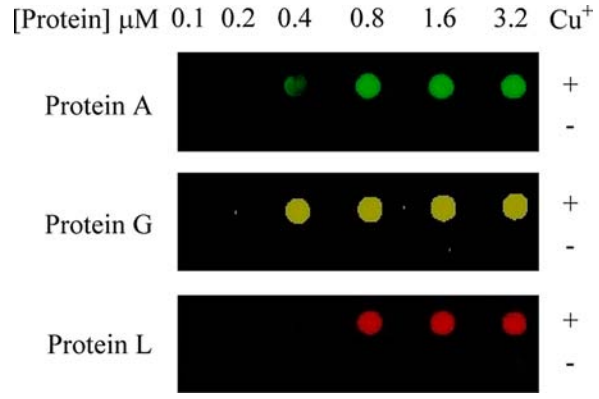

Figure 1. Images of wells containing proA-CVIApf, proG-CVIApf, and proL-CVIApf treated with 1 μg/mL of the following fluorescent antibodies: proA (green, Alexa 488-labeled rabbit anti-GFP IgG), proG (yellow, Alexa 555-labeled goat anti-human IgG), and proL (red, Texas Red-labeled monoclonal mouse anti-GFP IgG). Wells were treated with (+) and without (−) Cu(I). Fluorescence intensities were measured by excitation/detection at 532/526 (Protein A), 532/555 (Protein G), and 532/580 nm (Protein L).

identical and in many cases are substantially different. In contrast protein L binds to the V_L region of κ-light chains and, as a result, does not bind to those antibodies without κ-light chains. ProA-CVIApf and proG-CVIApf were immobilized in different wells of a matted slide. The silicone mat was removed, and the slide was incubated with Alexa 555-labeled goat anti-human IgG, which binds more strongly to protein G than to protein A.[40] The slide was washed, and fluorescence intensities were measured with excitation/detection at 532/555 nm (Figure 2). Wells containing proG-CVIApf gave strong signals, while those containing proA-CVIApf did not. The slide was washed and then incubated with Alexa 488-labeled rabbit anti-GFP IgG, which binds to both proteins. After the slide was washed, strong signals were seen for all of the wells. No fluorescence was seen for control wells where Cu(I) was not

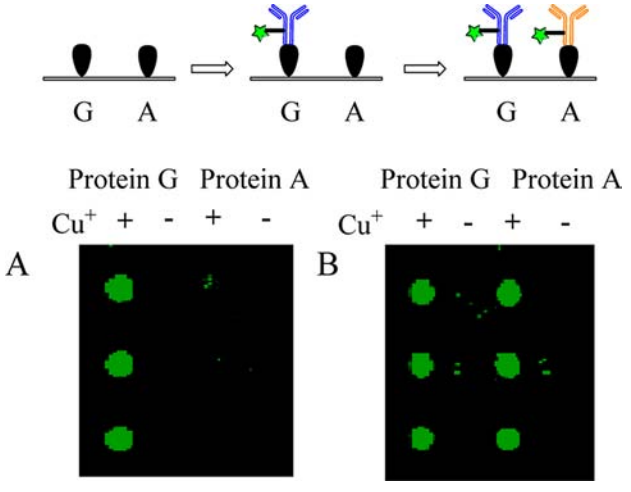

Figure 2. Fluorescence images for proA-CVIApf and proGA-CVIApf immobilized on the same slide. Incubation with (A) Alexa 555-labeled goat anti-human IgG (proG-CVIApf) and (B) Alexa 488-labeled rabbit anti-GFP IgG (proA-CVIApf and proG-CVIApf). Fluorescence intensities were measured by excitation/detection at 532/555 nm. The same conditions for a slide prepared with (+) and without (−) Cu^+ in the immobilization buffer.

included in the immobilization buffer. These results demonstrate the combination of selective and overlapping affinities of proA-CVIApf and proG-CVIApf for different antibodies. In general, protein G binds more strongly to a larger selection of antibodies than protein A, while protein A is more selective for detecting different subclasses of mouse and human IgG.[41]

ProG-CVIApf and proL-CVIApf were immobilized in individual wells and visualized with a mixture of Alexa 680-labeled goat anti-rabbit IgG, which binds to protein G and not protein L, and Alexa 488-labeled mouse IgM, which binds to protein L and not protein G. As shown in Figure 3, excitation at

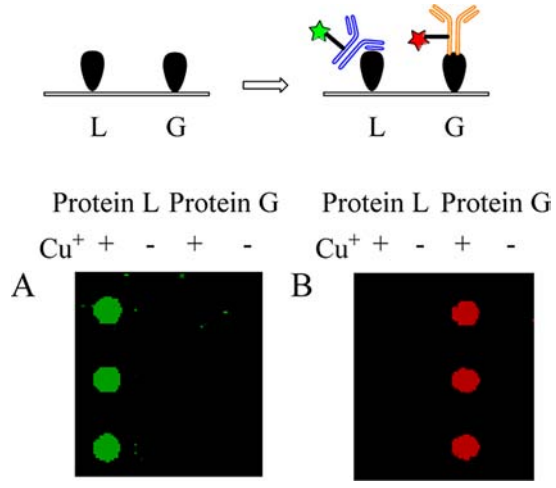

Figure 3. Fluorescence images of immobilized proG-CVIApf and proL-CVIApf on the same slide. The slides were incubated with a mixture of fluorescent antibodies, Alexa 488-labeled mouse IgM (green) and Alexa 680-labeled goat anti-rabbit IgG (red). Fluorescence intensities were measured each at 532 (A) and 633 nm (B); (+) in the presence of Cu(I) or (−) in the absence of Cu(I).

532 nm gave strong signals for the proL-CVIApf wells, while excitation at 633 nm gave strong signals for the proG-CVIApf wells. No signals were seen for controls where Cu(I) was not included in the immobilization buffer. Thus, recombinant proG-CVIApf and proL-CVIApf show high selectivity for their respective antibodies, as expected from the differences in their binding regions.

Finally, a slide was prepared where proA-CVIApf, proG-CVIApf, and proL-CVIApf were immobilized in individual wells. The mat was removed and the slide was incubated with a mixture of Alexa 680-labeled goat anti-rabbit IgG and Alexa 488-labeled mouse IgM. After the slide was washed, fluorescence intensities were measured each at 532 and 633 nm. Alexa 680-labeled goat anti-rabbit IgG showed strong emission at 670 nm for the proG-CVIApf wells, while the proA-CVIApf and proL-CVIApf wells did not show any signals (Figure 4). Intense fluorescence of mouse IgM was seen at 532 nm for the wells containing proL-CVIApf, but not for those containing proA-CVIApf and proG-CVIApf. These results emphasize the high selectivity of the recombinant antibody-binding proteins.

Binding Capacity of Immobilized proA-CVIApf, proG-CVIApf, and proL-CVIApf. To estimate the amount of antibody-binding proteins on the surface of the slides, we coated the entire surface of slides with proA-CVIApf, proG-CVIApf, or proL-CVIApf. A coverslip was placed on the azido-derivatized surface, and a solution of proA-CVIApf, proG-

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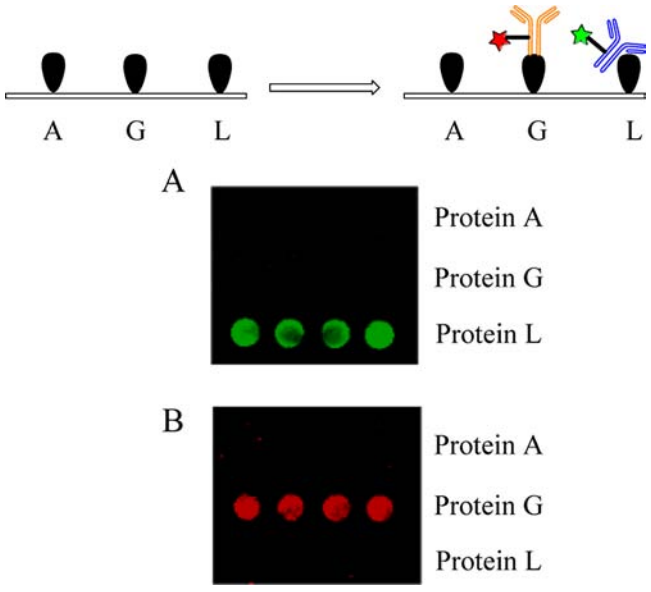

Figure 4. Fluorescence images of immobilized proA-CVIApf, proG-CVIApf, and proL-CVIApf on the same slide. The slide was incubated with a mixture of fluorescent antibodies, Alexa 680-labeled goat anti-rabbit IgG (0.2 μg/mL, red) and Alexa 488-labeled mouse IgM (1 μg/mL, green). Fluorescence intensities were measured at 532 (A) and 633 nm (B).

CVIApf, or proL-CVIApf in click buffer was added along the edge of the coverslip. The slides were gently shaken for 4 h in a humid hybridization chamber at rt. The coverslips were removed, the slides were washed with PBST, blocking solution was added to the chamber, and the slides were incubated overnight at 4 °C. Silicone mats were attached, and a series of concentrations of the appropriate fluorescent antibodies were spotted into the wells. After incubation overnight at 4 °C, the slides were washed twice with PBST at rt. A plot of fluorescence intensity versus concentration indicates that the surfaces were saturated with bound antibodies when 7 μL of ~20 μg/mL solutions was applied (see Figure 5).

Stacked Binding Arrays. Enzyme-linked immunosorbent assay (ELISA) techniques are widely used to detect and quantify antigens adsorbed on the surface of a microtiter plate.[42] The "sandwich" modification uses surface-bound capture antibodies to improve antigen binding in cases where the antigen is poorly adsorbed.[43] This technique is sometimes difficult to miniaturize because the capture antibody may be poorly adsorbed in random orientations. We explored the use of antibody-binding proteins to tightly bind and orient the capture antibody for antigen binding and detection. For this approach to be useful for quantitative applications, the antibody-binding protein must selectively bind the capture antibody and not the detection antibodies. A proL-CVIApf-coated slide provides a surface for chemo- and regioselective binding of the capture antibody without competing binding for the surface by the detection antibody. Protein L binds the kappa light chains of antibodies except those from goats, cows, and sheep.[44] Rabbit antibodies are weakly bound. Thus, cross-reactivity can be avoided by using a slide coated with protein L in combination with antibodies from other organisms for capture and antibodies from goats, cows, and sheep for detection.

We used the sandwich procedure to detect green fluorescent protein (GFP) in solution and selectively detect GFP and

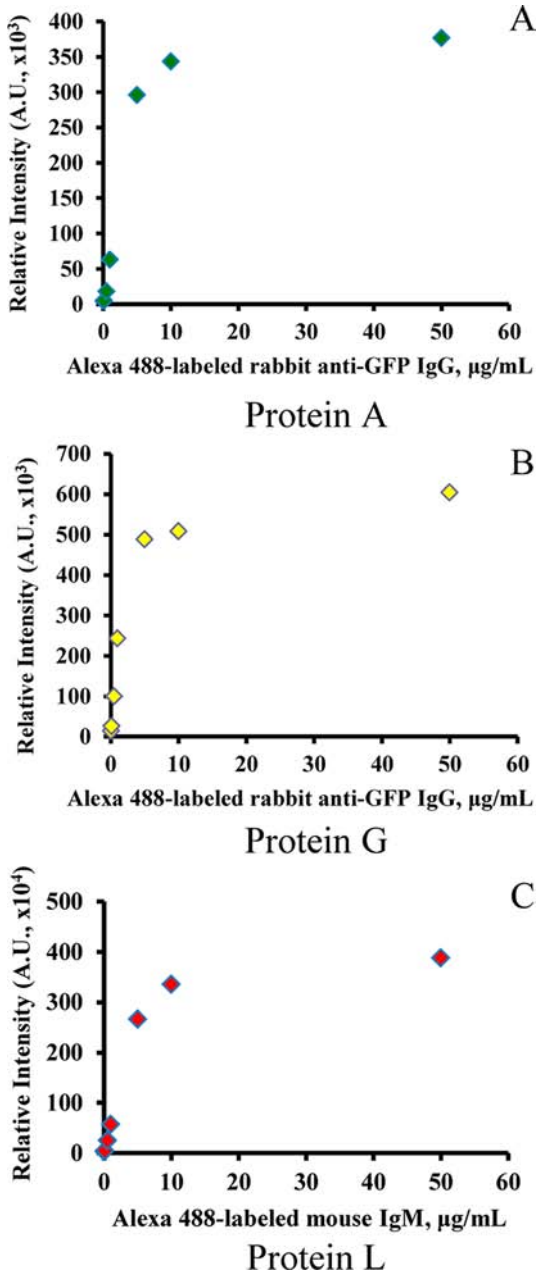

Figure 5. Immobilization of proA-CVIApf, proG-CVIApf, and proL-CVIApf. The slides were incubated with fluorescent antibodies as follows: proA-CVIApf (green, Alexa 488-labeled rabbit anti-GFP IgG, A), proG-CVIApf (yellow, Alexa 488-labeled rabbit anti-GFP IgG, B), and proL-CVIApf (red, Alexa 488-labeled mouse IgM, C). Fluorescence intensities were measured at 532 nm.

glutathione S-transferase (GST) in a mixture of the two proteins with mouse monoclonal anti-GFP IgG and mouse monoclonal anti-GST IgG as the capture antibodies. Each binding step in the GFP assay was investigated, starting with addition of mouse anti-GFP IgG to a slide coated with proL-CVIApf. Different concentrations of Texas Red-labeled mouse monoclonal anti-GFP were spotted into wells of a matted surface-coated proL-CVIApf slide. As shown in Figure 6A, a 10 μg/mL solution of fluorescent Texas Red-labeled mouse monoclonal anti-GFP IgG was sufficient to saturate the surface of wells on a matted slide. A slide was then prepared with unlabeled mouse monoclonal anti-GFP IgG as the capture

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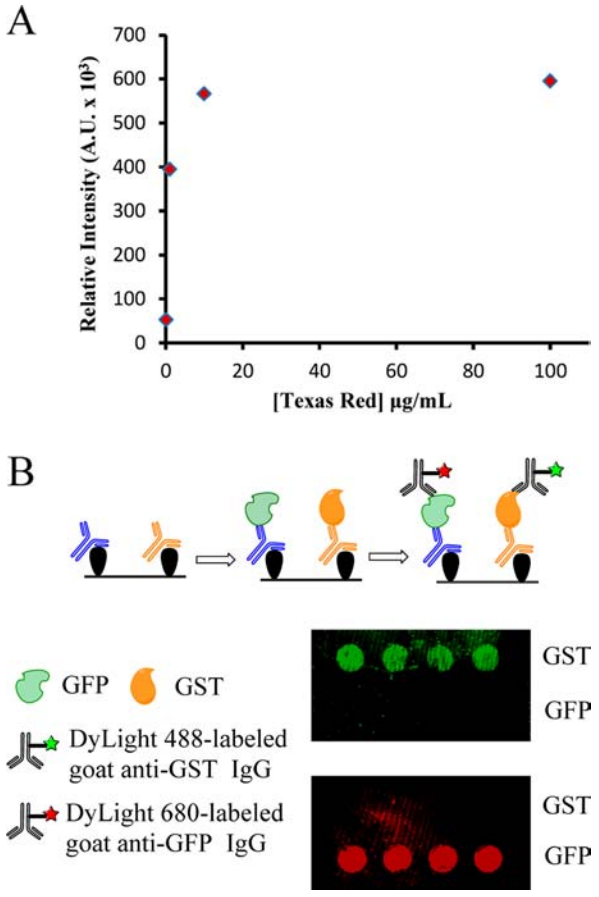

Figure 6. Detection of GFP and GST by a sandwich array on a proL-CVIAfp slide. (A) Detection of capture antibody with different concentrations of Texas Red-labeled mouse monoclonal anti-GFP IgG. (B) Selective detection of GST and GFP. Fluorescence images of a slide with mouse monoclonal anti-GFP IgG (blue) and mouse monoclonal anti-GST IgG (orange) spotted in individual wells as capture antibodies, followed by successive incubations with a mixture of GFP and GST and a mixture of DyLight 680-labeled goat anti-GFP IgG (red) and DyLight 488-labeled goat anti-GST IgG (green) with excitation at 532 and 633 nm. Details are presented in the SI.

antibody. Addition of GFP gave concentration-dependent weakly fluorescent spots upon excitation at 532 nm and intensely fluorescent spots with developed with DyLight 680-labeled goat anti-GFP IgG (Figure 6B).

Finally, mouse monoclonal anti-GFP IgG (20 μg/mL) and mouse monoclonal anti-GST IgG (20 μg/mL) were added to individual wells of a slide coated with proL-CVIApf. The silicone mat was removed, and the slide was incubated with a mixture of GFP and GST. The slide was washed thoroughly and then incubated with a solution containing DyLight 680-labeled goat anti-GFP IgG and DyLight 488-labeled goat anti-GST IgG. Visualization at 532 nm gave intense spots for GST in the wells containing mouse monoclonal anti-GST IgG, while visualization at 633 nm gave intense spots for GFP in the wells containing mouse monoclonal anti-GFP IgG. We did not see any evidence for cross-reactivity. Thus, it should be straightforward to use a single proL-CVIApf-coated slide for multiple analyses.

Regeneration of Slides. Since antigen−antibody complexes are disrupted at low pH, we examined the possibility of regenerating our slides after they had been used to detect fluorescent antibodies.[45] If successful, this would potentially extend the useful life of the slide and permit it to be used in applications with a variety of antibodies and ligands. A proA-CVIApf-coated slide was prepared as described above. Three different concentrations of rabbit anti-goat IgG were added to different wells. The slide was incubated, and the mat was removed and washed with PBST buffer. The slide was then incubated with Alexa 488-labeled goat anti-human IgG overnight and again washed. Fluorescence intensities were measured at 532 nm. The slide was then treated with glycine·HCl buffer, followed by treatment with PBS buffer. The mat was reapplied, and rabbit anti-goat IgG was added as before. The slide was incubated at 4 °C overnight and washed twice with PBST. The slide was incubated again with Alexa 488-labeled goat anti-human IgG, and fluorescence was measured at 532 nm (Figure 7). These experiments demonstrate the ability to strip bound antibodies from the slide and regenerate the surface for a second round of binding.

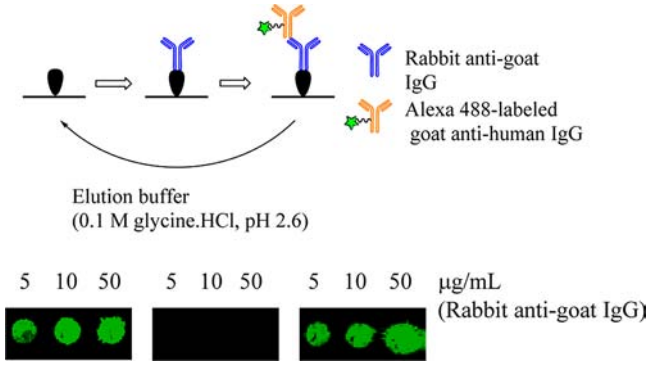

Figure 7. Regeneration of a proA-CVIApf slide. Rabbit anti-goat IgG (blue) and Alexa 488-labeled goat anti-human IgG (orange). Fluorescence intensities were measured at 532 nm.

CONCLUSIONS

A protein farnesyltransferase C-terminal CVIA motif was appended to truncated versions of antibody-binding proteins A, G, and L, and the cysteine residue in the motif of the resulting recombinant proteins was regioselectively modified with an analogue of farnesyl diphosphate bearing an propargyl moiety in the ω-position. The proteins were linked to a glass slide, whose surface was coated with covalently attached PEG linkers bearing an azide moiety, by a Cu(I)-catalyzed Huisgen cycloaddition. The farnesylation and cycloaddition reactions are bioorthogonal, and, as a result, both were carried out with the crude cell-free homogenates from the *E. coli* expression vectors without the need to purify the antibody-binding proteins. The covalently anchored antibody-binding proteins showed the same selectivity as the wild-type proteins for binding of a variety of fluorescently tagged antibodies. The surfaces of the slides were saturated at applied antibody concentrations of 10−20 μg/mL The antibody-binding proteins can also be used in "sandwich" applications by taking advantage of their selectivity for antibody binding, as demonstrated by detection of glutathione S-transferase using a protein L slide to bind mouse monoclonal anti-GST IgG as the capture antibody and goat anti-GST IgG as the detection antibody. Finally, the covalently coated slides can be stripped and reused.

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■ ASSOCIATED CONTENT

Supporting Information

Additional experimental details regarding PCR primers; general method for selective binding of recombinant antibody-binding proteins; stacked binding arrays with antibody-binding protein L-CVIApf; binding characteristics of Protein A, Protein G, and Protein L (Table S1); SDS-PAGE and Western blots of proteins A-CVIA, G-CVIA, L-CVIA, A-CVIApf, G-CVIApf, and L-CVIApf (Figure S1); fluorescence intensity of immobilized proteins A-CVIApf, G-CVIApf, and L-CVIApf (Figures S2); fluorescence images of immobilized proteins A-CVIApf, G-CVIApf, and L-CVIApf on the entire surface by coverslip (Figure S3); fluorescence images of immobilized Protein L-CVIApf (Figures S4); and mass spectra of purified Protein A-CVIA, Protein A CVIApf, Protein G-CVIA, Protein G-CVIApf, Protein L-CVIA, and Protein L-CVIApf (Figures S5). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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