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Sandwich Antibody Arrays Using Recombinant Antibody-Binding Protein L

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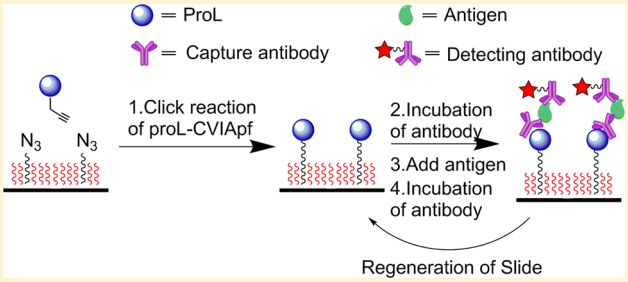
Supporting Information

ABSTRACT: Antibody arrays are a useful for detecting antigens and other antibodies. This technique typically requires a uniform and well-defined orientation of antibodies attached to a surface for optimal performance. A uniform orientation can be achieved by modification of antibodies to include a single site for attachment. Thus, uniformly oriented antibody arrays require a bioengineered modification for the antibodies directly immobilization on the solid surface. In this study, we describe a "sandwich-type" antibody array where unmodified antibodies are oriented through binding with regioselectively immobilized recombinant antibody-binding protein L. Recombinant proL-CVIA bearing C-terminal CVIA motif is post-translationally modified with an alkyne group by protein farnesyltransferase (PFTase) at the cysteine residue in the CVIA sequence to give proL-CVIApf, which is covalently attached to an azido-modified glass slide by a Huisgen [3 + 2] cycloaddition reaction. Slides bearing antibodies bound to slides coated with regioselectively immobilized proL-CVIApf gave stronger fluorescence outputs and those where the antibody-binding protein was immobilized in random orientations on an epoxy-modified slide. Properly selected capture and detection antibodies did not cross-react with immobilized proL-CVIApf in sandwich arrays, and the proL-CVIApf slides can be used for multiple cycles of detected over a period of several months.



INTRODUCTION

Microarrays provide a sensitive approach for detecting a wide range of small molecules, biopolymers, and cells with important analytical applications in medicine and the environment.[1−4] These techniques have been widely applied by miniaturization of conventionally analytical devices.[5] Antibody-based assays,[6] for example ELISA (enzyme-linked immunosorbent assay), are especially useful for detection and quantification of antigens.[7] High sensitivity is important for fluorescence-based high throughput screening of antigens or antibodies on a biosensor. The sensitivity and stability of protein-based micorarrays are limited by whether the proteins are immobilized covalently or noncovalently, the orientation of the immobilized proteins on the surface, and the ability of the proteins to retain their native conformations during and after immobilization.[8−10]

Antibody-binding proteins A, G, and L (proA, proG, and proL) have received considerable attention for construction of protein microarrays suitable for immobilization of intact antibodies.[11−14] Recently, we reported construction of glass slides where their surfaces were coated with proA, proG, and proL attached regio- and chemoselectively.[15−17] Recombinant forms of proA, proG, and proL bearing a CVIA C-terminal recognition motif for protein farnesyltransferase (PFTase) were post-translationally modified with a farnesyl analogue containing an ω-terminal alkyne functional group to give proApf, proGpf, and proLpf, which have strong and stable binding affinities for various types of antibodies.[18,19] The antibody proteins were covalently attached to the surface of glass slides bearing a complementary azide linker by a Cu(I)-catalyzed Huisgen [3 + 2] cycloaddition reaction. The slides were constructed by applying cell free lysates of recombinant antibody-binding proteins proApf, proGpf, or proLpf that were post-translationally modified immediately after the cells were disrupted.

Although proA and proG are useful for binding of a variety of antibodies, the special binding behavior of proL makes it especially attractive as a foundation for antibody-based microarrays.[20,21] While proA and proG bind the Fc region of a large number of antibodies, proL binds to κ light chains of a more limited set and, in addition, does not interfere with the antigen-binding region of the antibody.[22−24] Sandwich-type antibody microarrays on protein chips use a capture antibody to bind an antigen on the surface and a detection antibody to detect the bound antigen. The strong binding of proA and proG to antibodies from cows, goats, mouse, rabbits, and sheep commonly used for detection in immunoassays can lead to false positives resulting from binding of the detection antibody to the antibody-binding protein.[25] In contrast, proL does not bind to cow, goat, and sheep antibodies and binds weakly to rabbit antibodies.[26] Thus, proL has two advantages as a capture

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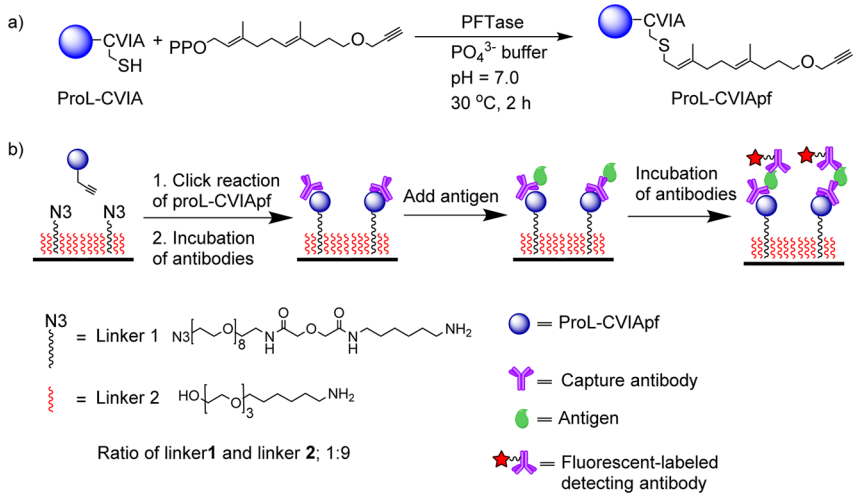
Scheme 1. Construction of a Sandwich Antibody Array



antibody. It provides more opportunities for minimizing false positives, and the capture antibody, which is bound through its κ light chain, has two exposed antigen binding sites. We now report the construction of antibody-binding sandwich structures based on proL covalently attached to silica surfaces.

EXPERIMENTAL SECTION

Slides with ProL-CVIApf Immobilized in Wells. An azido-modified glass slide was rinsed with phosphate-buffered saline (PBS, pH 7.4) and deionized water and dried under N_2. A silicone mat (Sigma, S3810) was attached, and 1.5 μL samples of proL-CVIApf were added to individual wells, followed by 3.5 μL of "Huisgen buffer" (see Supporting Information) to give final concentrations of 0.001–100 μM. The slide was placed in a hybridization chamber and shaken (100 rpm) for 2 h at room temperature (rt). The silicone mat was removed, and the slide was washed twice with PBST (PBS buffer containing 0.1% Tween 20, pH 7.2) at rt in a high throughput wash station. Blocking solution (1% bovine serum albumin (BSA) in PBST, 3 mL) was added, and the slide was shaken for 1 h at rt. The slides were washed twice with PBST at rt and stored in PBS with 0.01% (w/v) NaN_3 at 4 °C until needed.

Slides with ProL-CVIApf Immobilized on the Entire Surface. An azido-modified glass slide was rinsed with PBS and deionized water and dried under N_2. The slide was covered with an mSeries LifterSlip coverslip, and 55 μL of a solution prepared by mixing 30 μL of 333 μM proL-CVIApf with 70 μL of "Huisgen buffer" was inserted at the edge of the coverslip. The slide was shaken at 70 rpm for 2 h at rt, the coverslip was removed, and the slide was washed twice with PBST in the wash station. Blocking solution (3 mL) was added to the chamber, and the slide was shaken for 1 h at rt. The slide was washed with PBST in the wash station and stored in PBS with 0.01% (w/v) NaN_3 at 4 °C until needed.

Surface-Coated ProL-CVIA Slides with Bound Mouse Anti-GFP IgG. ProL-CVIApf coated slides were prepared as described above. The slide was washed with PBS, dried under N_2, and fitted with an mSeries LifterSlip coverslip. Mouse monoclonal anti-GFP (55 μL, 1 mg/mL) was inserted at the edge of the coverslip. The slide was shaken at 70 rpm for 2 h at rt, and the coverslip was carefully removed. The slide was washed twice with PBST in the wash station and stored in PBS with 0.01% (w/v) NaN_3 at 4 °C until needed.

Stripping Bound Antibodies. Slides incubated with fluorescent-labeled antibodies were treated with 3 mL of stripping buffer (0.1 M glycine, pH 2.6; 0.1 M glycine, pH 10.0, 1% SDS or 3.5 M $MgCl_2$) for 40 min. After washing with PBST at rt, the slides were scanned with a Typhoon 8600 phosphoimager. Slides stripped with 0.1 M glycine, pH 2.6, were immediately treated with 3 mL of 1 M Tris.HCl, pH 8.5, for 10 min. The slides were washed twice with 0.2% Triton X-100 at rt for 10 min to remove nonspecifically bound protein, if needed. The slides were washed twice with PBST at rt and stored at 4 °C in the PBST with 0.01% (w/v) NaN_3 until needed.

Visualization of Fluorescent-Labeled Antibodies and Green Fluorescent Protein (GFP). Fluorescent antibodies were diluted to the appropriate concentrations in PBS (0.5–1.0 μg/mL in 3 mL) before use. Slides were incubated at rt for 1 h with shaking at 60–90 rpm, washed twice with PBST at rt in the wash station, and dried under N_2. Fluorescence intensities were measured at excitation/detection at 532/526 (Alexa 488 and GFP), 633/670 (Alexa 680 and DyLight 680), and 532/580 (Texas Red) nm. All fluorescence intensities were measured with a Typhoon 8600 phosphoimager.

RESULTS AND DISCUSSION

Construction of Sandwich Arrays. *Peptostreptococcus magnus* antibody-binding proL is a 109 kDa protein with antibody-binding domains, B1–B5, that bind to κ light chains of antibodies without interfering with their antigen-binding regions.[27,28] ProL has been used to purify and detect antigens or antigen–antibody complexes by immunoprecipitation (IP).[29,30] In a previous study, we constructed a truncated form of proL, proL-CVIA (35.2 kDa), that contains the B1–B4 antibody-binding domains and a C-terminal CVIA recognition motif for protein farnesyltransferase.[15] The protein was post-translationally modified at the cysteine residue using a farnesyl diphosphate analogue with an ω-terminal alkyne residue to give proL-CVIApf (Scheme 1a). A complementary azido-derivatized glass slide was prepared by constructing self-assembled monolayers (SAMs) using of 1:9 molar ratio of polyethylene glycol (PEG) linkers bearing a terminal azide moiety (Linker **1**) and a hydroxyl group (Linker **2**). ProL-CVIApf was attached covalently to the azido-derivatized glass slide by a Cu^+-catalyzed Huisgen [3 + 2] cycloaddition.[31,32]

Sandwich antibody[33] arrays were constructed using a monoclonal mouse antibody for capture and a polyclonal goat antibody for to prevent overlapping epitopes in the capture and detection antibodies. Immobilized proL-CVIApf strongly binds mouse antibodies but does not bind those from goats. Green fluorescent protein (GFP) was chosen as a fluorescent antigen. Fluorescence intensities of GFP and DyLight 680-labeled antibodies were measured by excitation/detection at 532/526 and 633/670 nm, respectively. This construct is illustrated in Scheme 1b.

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Detection Antibodies Do Not Bind to Immobilized ProL-CVIApf. It is important that the detection antibody not bind to the antibody-binding protein in order to prevent false positives in a sandwich assay using proL-CVIA to bind the capture antibody. As a control, wells of matted glass slide surface coated with proL-CVIAfp were treated with 0.01−100 μg/mL mouse anti-goat IgG as a capture antibody. The mat was removed, and the slide was incubated with Alexa 680-labeled goat anti-rabbit IgG. The absence of background fluorescence in Figure 1a demonstrates that proL-CVIApf did

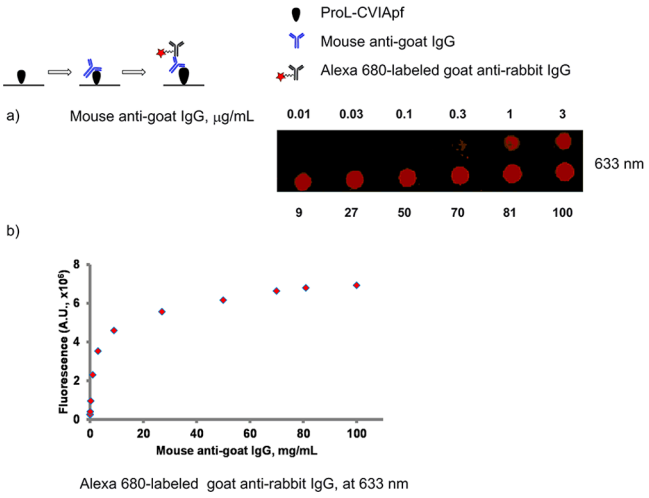

Figure 1. Specific capture-detection antibody binding on proL-CVIApf coated slides. ProL-CVIApf coated slide was incubated with mouse anti-goat IgG (0.01−100 μg/mL) followed by treatment with Alexa 680-labeled goat anti-rabbit IgG (1.0 μg/mL). Part a: fluorescence intensities measured at excitation/detection 633/670 nm (Alexa 680-labeled goat anti-rabbit IgG, Red). Part b: plots of relative fluorescence intensities versus the concentration of mouse anti-goat IgG.

not bind to the fluorescent-goat IgG. In addition, the wells treated with mouse anti-goat IgG as a capture antibody gave dose-dependent signals at excitation/emission 633/670 nm for Alexa 680-labeled goat anti-rabbit IgG. The plots of relative fluorescence intensity versus capture antibody concentration (Figure 1b) indicate that the proL-CVIApf binding sites are saturated when incubated with 100 μg/mL solutions of the capture antibody. In addition, the fluorescent signal is specifically dependent on binding between the capture and detection antibodies.

Detection of GFP with a Fluorescent Sandwich Array. Sandwich structures for detecting GFP were constructed on glass slides and evaluated for their sensitivity and selectivity. The layers of the sandwich consisted of proL-CVIApf, used to anchor the mouse anti-GFP IgG capture antibody, GFP, and the DyLight 680-labeled mouse anti-GFP IgG detection antibody. Three experiments were used to evaluate the sensitivity and cross-reactivity of the protein components used in the array (Scheme S1).

In the first experiment, 0.001, 0.01, 0.1, 1.0, 10, and 100 μM samples of proL-CVIApf in PBS containing "Huisgen buffer" were added to wells of a matted azido-modified glass slide. The mat was removed, and the slide was incubated with excess mouse monoclonal anti-GFP IgG using the coverslip technique, which minimizes the amount of anti-GFP IgG needed to saturate the available proL-CVIApf binding sites. The slide was transferred to a chamber containing 20 μM GFP in PBS buffer, was washed with PBS, and was visualized. Weakly fluorescent spots were observed for GFP (Figure S1a). The slide was then incubated with DyLight 680-labeled goat anti-GFP IgG (1 μg/mL) and visualized to give a series of spots whose intensities increased with increasing concentrations of proL-CVIApf used for immobilization of the antibody-binding protein (Figure 2a).

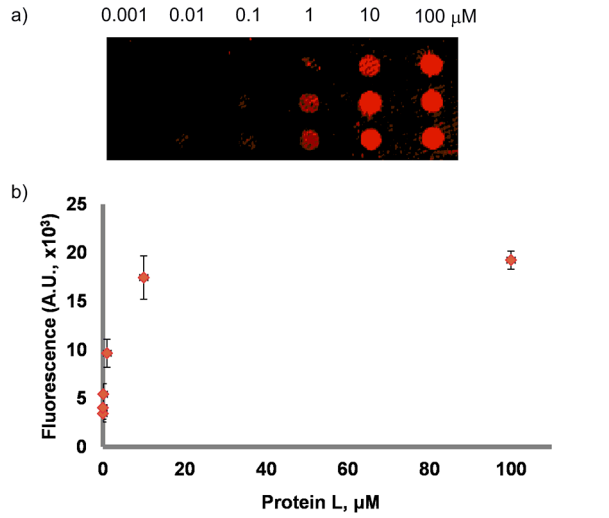

Figure 2. Variation of [proL-CVIApf]. Slides were treated with 0.001−100 μM proL-CVIApf at rt for 2 h and subsequently incubated in sequence with mouse anti-GFP IgG (55 μL, 1 mg/mL), GFP (20 μM), and DyLight 680-labeled goat anti-GFP IgG (1.0 μg/mL). Part a: fluorescence intensities measured at excitation/detection 532/526 nm (GFP) or 633/670 nm (DyLight 680-labeled goat anti-GFP IgG). Part b: plot of relative fluorescence intensity versus [proL-CVIApf].

A plot of fluorescence intensity versus the concentration of proL-CVIApf used in the immobilization reaction indicates that ∼1 μM proL-CVIApf modifies half of the available azide sites, and all of the available sites are modified with 10 μM proL-CVIApf (Figure 2b). It is possible that some of the azide groups are sterically blocked by surface bound proL. These results are similar to our previous studies where we established that covalent attachment of proteins bearing a C-terminal CVIApf motif requires Huisgen buffer and measured the binding capacity of the azide modified slides.[15−17]

In the second experiment, the entire surface of the slide was modified with proL-CVIApf. A mat was applied, and 0.001, 0.01, 0.1, 1.0, 10, and 100 μg/mL samples of mouse anti-GFP IgG were added to individual wells. The mat was removed, and the slide was washed with PBS. The slide was treated with PBS containing 20 μM GFP, washed, and visualized to give a series of weakly fluorescent spots (Figure S1b). The slide was then incubated with DyLight 680-labeled goat anti-GFP IgG (1 μg/mL) and visualized to give a series of spots whose intensities increased with increasing concentrations of the capture antibody (Figure 3).

A plot of fluorescence intensity versus the concentration of mouse anti-GFP IgG indicates that ∼10 μg/mL of the capture antibody is sufficient to occupy the available proL-CVIA binding sites (Figure 3b). A comparison of AFM images for the surfaces of amine, succinimidyl ester, and azido-PEG modified slides with a slide modified with proL-CVIApf indicates good coverage by the covalently immobilized protein (Figure S2).

Finally, different concentrations of GFP in PBS were detected using a slide coated with proL-CVIApf with mouse

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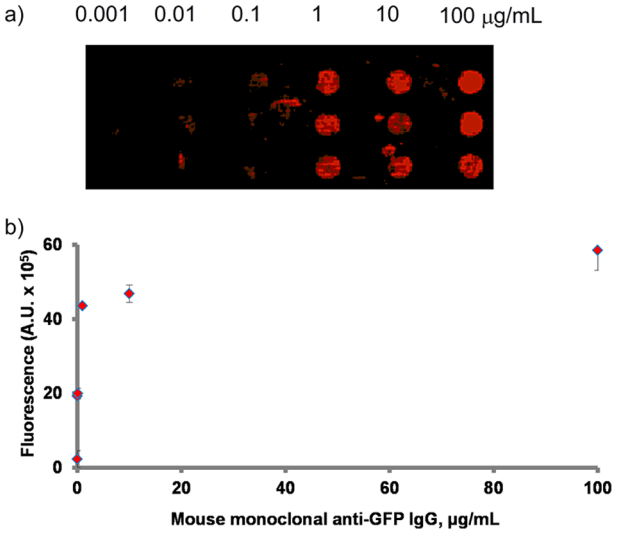

Figure 3. Variation of [mouse anti-GFP IgG]. Slides were treated with 100 μM proL-CVIApf at rt for 2 h and incubated in sequence with mouse anti-GFP IgG (10 μL, 0.001−100 μg/mL), GFP (20 μM), and DyLight 680-labeled goat anti-GFP IgG (1.0 μg/mL). Part a: fluorescence intensities measured at excitation/detection 532/526 nm (GFP) and 633/670 nm (DyLight 680-labeled goat anti-GFP IgG). Part b: plot of relative fluorescence intensity versus [mouse anti-GFP IgG].

anti-GFP IgG capture and DyLight 680-labeled goat anti-GFP IgG detection antibodies. Solutions of GFP (0.001−100 μM) were added to individual wells of the matted slide, which had been previously treated with the capture antibody. The slide was washed, the mat was removed, and GFP fluorescence was detected (excitation/emission at 532/526 nm; see Figure S1c). The slide was then incubated with DyLight 680-labeled goat anti-GFP IgG and visualized (excitation/emission at 633/670 nm, Figure 4a). Fluorescent spots were easily seen for well spotted with 10 nM GFP. The high signal-to-noise ratio can be

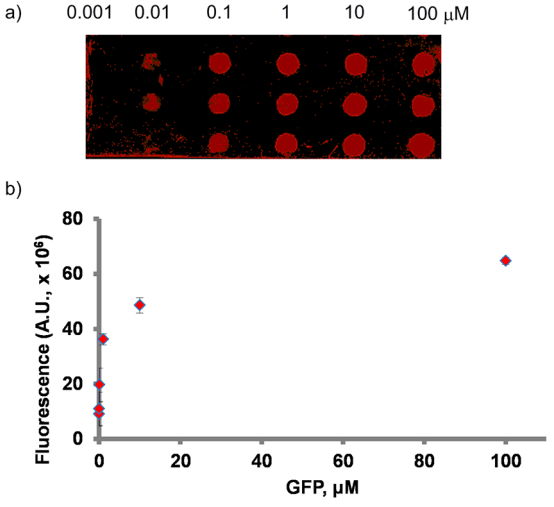

Figure 4. Detection of GFP with a sandwich-type antibody array. A slide was surface-coated with 100 μM proL-CVIApf and incubated in turn with mouse anti-GFP IgG (55 μL, 1 mg/mL), GFP (10 μL, 0.001, 0,01, 0.1, 1.0, 10, and 100 μM), and DyLight 680-labeled goat anti-GFP IgG (1.0 μg/mL). Part a: fluorescence with excitation/detection at 633/670 nm (DyLight 680-labeled goat anti-GFP IgG). Part b: plot of fluorescence intensity versus [GFP].

attributed to the absence of cross-reactivity among surface bound proL-CVIApf, the capture antibody and the detection antibody, and the absence of nonspecific binding of the detection antibody to the PEG monolayer on the surface of the slide.

Comparison of Sandwich on Slides with Oriented and Randomly Attached Antibodies. Slides or chips with surfaces bearing aldehyde,[34] epoxy,[35] or *N*-hydroxysuccinimidyl ester[36] functional groups are commonly used to immobilize proteins covalently through reactions with nucleophilic functional groups in amino acid side chains on the protein's surface. However, the proteins are often attached through different nucleophiles in a variety of different orientations, some of which may interfere with the function of the immobilized protein and lower the sensitivity of the assay.[37] In addition, interactions with the solid surface of adsorbed can result in denaturation.[38,39] Protein denaturation can be reduced by polyethylene glycol (PEG) linkers that separate the immobilized proteins from the surface. We compared immunoassays for covalently immobilized proL-CVIApf on a commercially available epoxy-modified slide with our slides coated with a mixture of PEGs terminated with azide and hydroxyl groups.

ProL-CVIApf (0.1−100 μM) was immobilized on both epoxy- and azido-modified glass slides. Mouse monoclonal anti-goat IgG and Alexa 680-labeled goat anti-rabbit IgG were added consecutively. The slides were washed with PBS buffer and visualized. The fluorescence intensities of azido-derivatized slide were slightly higher than those of epoxy-modified slide (Figure 5a). The slides were stripped with 0.1 M glycine at pH 2.6 for 40 min, the antibodies were reapplied, and the slides were visualized. The fluorescence intensities of epoxy-modified slide decreased by approximately 50% relative to those of azido-modified slide for the wells coated with 10 and 100 μM proL-CVIApf. These results indicate that proL-CVIApf was covalently attached to and physically absorbed on the surface of the epoxy-modified slide following a wash with PBST buffer. The lower fluorescence intensity for covalently attached protein after stripping the slide indicated a lower surface density, suboptimal orientations, or partial denaturation of proL on the epoxy slides relative to the azide slides. The physically bound proteins on the epoxy-modified surface were not completely removed by washing with PBST, as indicated by the decrease in fluorescence intensity relative to the azide slide following treatment with stripping buffer.

Regeneration of Slides. Immunoprecipitation (IP) or co-immunoprecipitation (Co-IP) with antibody-binding proteins A, G, and L on solid supports are useful techniques for purification of antibodies and antigen−antibody complexes.[40,41] These procedures can involve several hundred cycles of association and dissociation. We investigated the stability of our proL-CVIApf slides during multiple cycles of stripping, application of antibodies, and visualization using several different conditions known to disrupt interactions between biomolecules.

Developed and visualized proL-CVIApf-coated slides were stripped with and without detergent in buffers of different pHs and ionic strengths. The slides were developed with Texas Red-labeled monoclonal mouse anti-GFP IgG (1 μg/mL), stripped with 0.1 M glycine at pH 2.6, 0.1 M glycine at pH 10.0, 1% SDS, or 3.5 M $MgCl_2$, and redeveloped six times over a period of 4 months.[42] Fluorescence intensity (excitation/emission at 532/580 nm) was measured at the end of each cycle (Figure S3). The slides were stored at 4 °C in PBST buffer containing

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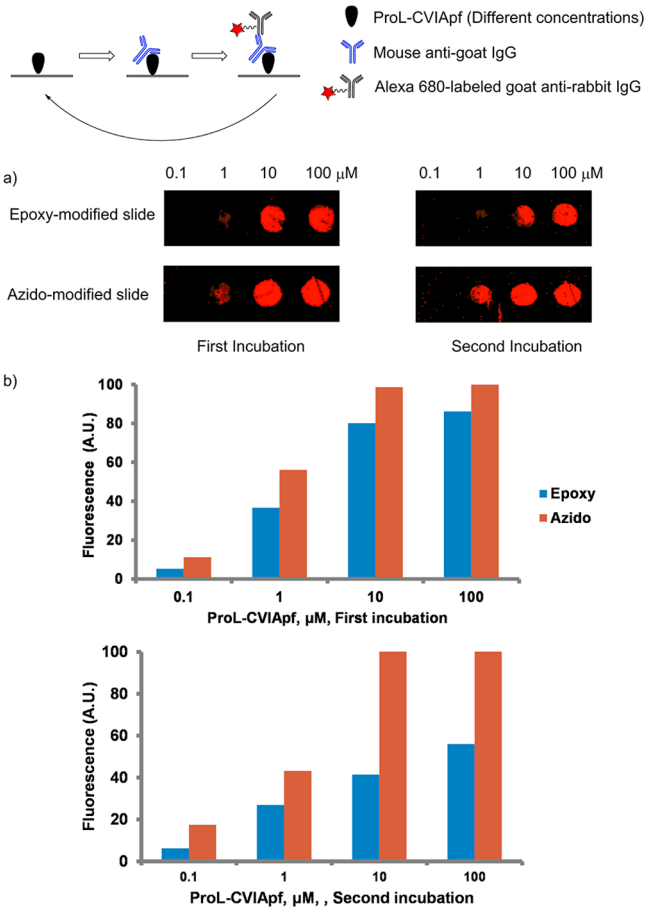

Figure 5. Comparison of epoxy-modified (blue) and an azido-modified (red) slides. Part a: slides were treated with 0.1−100 μM proL-CVIApf at rt for 2 h and incubated with mouse anti-goat IgG (55 μL, 1 mg/mL) and Alexa 680-labeled goat anti-rabbit IgG (1.0 μg/mL) at rt for 1 h. The slides were visualized and treated with Alexa 680-labeled goat anti-rabbit IgG (excitation/emission at 633/670 nm). The slides were treated with 0.1 M glycine at pH 2.6 for 40 min, and the treatment/visualization steps were repeated. Part b: plot of relative fluorescence intensity versus [proL-CVIApf].

0.01% NaN_3 between stripping cycles. Slides stripped under acidic conditions with 0.1 M glycine, pH 2.6, were neutralized by treatment with 1 M Tris·HCl, pH 8.5, before storage. Results for the most efficient regeneration system, 0.1 M glycine, pH 2.6, stripping buffer are shown in Figure 6. While stripping with 1% SDS is equally effective, proL-CVIApf looses binding capacity upon repeated treatment with the detergent.

ProL-CVIApf slides regenerated on six consecutive days with 0.1 M glycine, pH 2.6, buffer were used with mouse anti-GFP IgG capture antibodies in a sandwich-array for detecting GFP. Incubation with GFP (see Figure S4 for GFP visualization) was followed by treatment with DyLight 680-labeled goat anti-GFP IgG (1 μg/mL). A fluorescence standard was prepared by covalently attaching Alexa-680 succinimidyl ester directly to an azido-modified glass slide (Supporting Information). The sandwich complex was stripped from the slide upon treatment with 0.1 M glycine·HCl, pH 2.6, buffer, and the detection/stripping cycle was repeated six times. As shown in Figure 7 and Figure S5, the sensitivity of slides remained high during six cycles of regeneration.

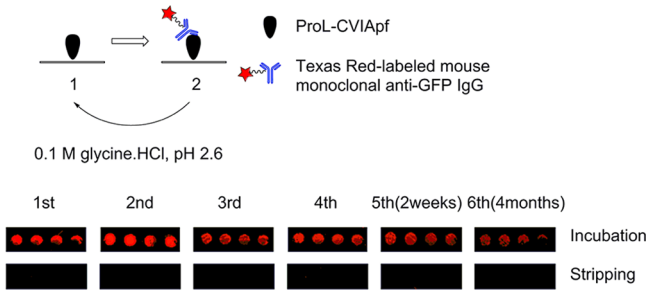

Figure 6. Regeneration of ProL-CVIApf-coated slides. Slides were treated with 10 μM μM proL-CVIApf at rt for 2 h and incubated with Texas Red-labeled monoclonal mouse anti-GFP IgG (1 μg/mL) at rt for 1 h. Fluorescence intensities were measured by excitation/emission at 532/580 nm.

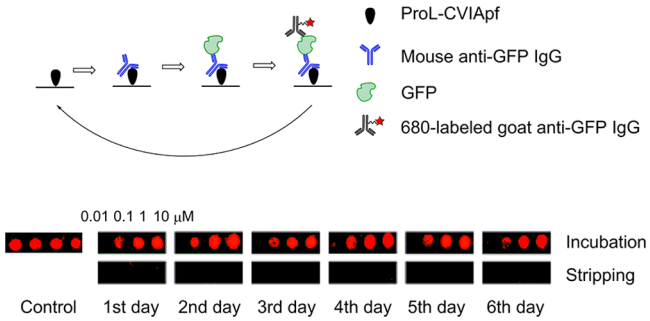

Figure 7. Multiple reuse of proL-CVIApf in sandwich-type antibody array. The slides were immobilized with 100 μM proL-CVIApf at rt for 2 h and subsequently incubated with mouse anti-GFP IgG (55 μL, 1 mg/mL), GFP (0.01−10 μM), and DyLight 680-labeled mouse anti-GFP IgG (1.0 μg/mL) at rt for 1 h. Fluorescence intensities were measured by each excitation/detection at 532/526 nm (GFP) and 633/670 nm (DyLight 680-labeled goat anti-GFP IgG).

CONCLUSION

Post-translationally modified recombinant protein L bearing a C-terminal alkyne moiety (proL-CVIApf) was covalently attached to PEG linkers on the glass slides by a Cu(I)-catalyzed Huisgen cycloaddition. Uniformly surface-coated proL-CVIApf slides capture antibodies for direct- and sandwich-type immunofluorescent detection of ligands in a microarray format. These slides gave low fluorescence backgrounds with the detection antibodies. The regioselective immobilization of proL-CVIApf on a hydroxyl-terminated PEG background optimizes the presentation of the proL-binding site and minimizes physical adsorption and denaturation during construction of the arrays. The proL-CVIApf slides can be regenerated and used for multiple cycles with the original or new capture and detection antibodies.

ASSOCIATED CONTENT

Supporting Information

Additional experimental details regarding post-translational modification of proL-CVIA; preparation of azido-modified glass slides; preparation of Huisgen buffer; immobilization of proL-CVIApf on epoxy-modified slides; preparation of standard for Alexa-680; AFM analysis of glass slides from each step in the immobilization of proL-CVIApf; fluorescent "sandwich" antibody array (Scheme S1); visualization of GFP in a sandwich antibody array (Figure S1); AFM images of modified slides and slide with proL-CVIApf (Figure S2); regeneration of proL-

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CVIApf slides (Figure S3); GFP fluorescence during regeneration (Figure S4); reuse multicycles of proL-CVIApf in "sandwich" antibody array (Figure 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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