Regio- and Chemoselective Immobilization of Proteins on Gold Surfaces

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Supporting Information

ABSTRACT: Protein chips are powerful tools as analytical and diagnostic devices for detection of biomolecular interactions, where the proteins are covalently or noncovalently attached to biosensing surfaces to capture and detect target molecules or biomarkers. Thus, fabrication of biosensing surfaces for regio- and chemoselective immobilization of biomolecules is a crucial step for better biosensor performance. In our previous studies, a regio- and chemoselective immobilization strategy was demonstrated on glass surfaces. This strategy is now used to regioselectively attach proteins to self-assembled monolayers (SAMs) on gold surfaces. Recombinant green fluorescent protein (GFP), glutathione S-transferase (GST), and antibody-binding protein G, bearing a C-terminal CVIA motif, were prepared and a farnesyl analogue with an ω-alkyne moiety was attached to the sulfhydryl moiety in the cysteine side chain by protein farnesyltransferase. The proteins, modified with the bioorthogonal alkyne functional group, were covalently and regioselectively immobilized on thiol or dithiocarbamate (DTC) SAMs on a gold surface by a Huigsen [3 + 2] cycloaddition reaction with minimal nonspecific binding. A concentration-dependent increase of fluorescence intensity was observed in wells treated with GFP on both thiol- and DTC-SAMs. The highly ordered, densely packed layer allowed for a high loading of immobilized protein, with a concomitant increase in substrate binding capacity. The DTC-SAMs were substantially more resistant to displacement of the immobilized proteins from the gold surface by β-mercaptoethanol than alkane-thiol SAMs.

INTRODUCTION

Biosensors or protein chips used in analytical and diagnostic devices are powerful tools for detection of biomolecular interactions and are becoming increasingly important in the field of biotechnology, where immobilized microarrays facilitate high-throughput analysis of protein–protein, protein–antibody, and protein–small molecule interactions.1–4 Typically, biomolecules such as antibodies or antigens deposited on a biosensing surface are used to capture and detect target molecules. For maximum sensitivity and reproducibility, the immobilized proteins should retain full activity and be oriented in random orientations that are not mobilized proteins. For maximum sensitivity and reproducibility, the biosensing surface are used to capture and detect target molecules or biomarkers. Thus, fabrication of biosensing surfaces for regio- and chemoselective immobilization of biomolecules is a crucial step for better biosensor performance. In our previous studies, a regio- and chemoselective immobilization strategy was demonstrated on glass surfaces. This strategy is now used to regioselectively attach proteins to self-assembled monolayers (SAMs) on gold surfaces. Recombinant green fluorescent protein (GFP), glutathione S-transferase (GST), and antibody-binding protein G, bearing a C-terminal CVIA motif, were prepared and a farnesyl analogue with an ω-alkyne moiety was attached to the sulfhydryl moiety in the cysteine side chain by protein farnesyltransferase. The proteins, modified with the bioorthogonal alkyne functional group, were covalently and regioselectively immobilized on thiol or dithiocarbamate (DTC) SAMs on a gold surface by a Huigsen [3 + 2] cycloaddition reaction with minimal nonspecific binding. A concentration-dependent increase of fluorescence intensity was observed in wells treated with GFP on both thiol- and DTC-SAMs. The highly ordered, densely packed layer allowed for a high loading of immobilized protein, with a concomitant increase in substrate binding capacity. The DTC-SAMs were substantially more resistant to displacement of the immobilized proteins from the gold surface by β-mercaptoethanol than alkane-thiol SAMs.

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naturally occurring functional groups, and whose binding properties are suitable for a wide variety of applications.\textsuperscript{25} In addition, the functionalized protein should be easy to prepare with minimal purification.

A technique initially described by Gauchet et al.\textsuperscript{26} fulfills these criteria for soluble proteins by introducing a bioorthogonal azide or alkyne moiety that can be coupled to a complementary alkyne or azide on the surface by a Huisgen [3 + 2] cycloaddition reaction. In this approach, a farnesyl diphasate derivative bearing an ω-alkyne or ω-azide moiety is attached to the sulfhydryl moiety of the cysteine residue in a C-terminal ‘CaaX’ recognition motif by protein farnesyltransferase (PFTase).\textsuperscript{27} The CaaX tetrapeptide, where “C” is cysteine, “a” is a small hydrophobic amino acid, and “X” is alanine, serine, methionine, or asparagine,\textsuperscript{28} can easily be appended by genetic engineering. Gauchet et al. demonstrated this immobilization strategy on glass surfaces for the Cu\textsuperscript{+} catalyzed Huisgen [3 + 2] cycloaddition and Staudinger reactions.\textsuperscript{29} This paper reports the adaptation of this approach for regioselective attachment of proteins to self-assembled monolayers on gold surfaces, which can be used in a variety of analyses, including surface plasmon resonance (SPR) and electrochemical applications.\textsuperscript{30}

\section{EXPERIMENTAL PROCEDURE}

\textbf{Preparation of ω-Azide SAMs.} ω-Azide alkanethiol SAMs were prepared on gold slides by swirling the slides at 100 rpm in ethanol containing 2 mM (total concentration) thiols 1 and 2 (see Scheme S1) for 44–48 h at rt. The coated slides were swirled three times, 15 min each, with ethanol, and dried with a gentle stream of N\textsubscript{2}.

ω-Azide dithiocarbamate SAMs were prepared on gold slides by swirling the slides at 100 rpm in ethanol containing 2 mM (total concentration) amines\textsuperscript{26,30} 3 and 4 (see Scheme 2) and 2 mM carbon disulfide for 44–48 h at rt. The coated slides were swirled three times, 15 min each, with ethanol and dried with a gentle stream of N\textsubscript{2}.

\textbf{Covalent Attachment of Modified Proteins to SAMs on Gold Slides.} A silicon mat was attached to a SAM-coated gold slide. A solution of GFP-CVIA, GST-CVIA, or proG-CVIA modified at the C-terminal cysteine with farnesyl-(GFP-CVIAf, GST-CVIAf, or proG-CVIApf) moieties was added to individual wells, followed by 0.5 mM CuSO\textsubscript{4}, 50 μM TBTA, 0.5 mM TCEP, and 50 μM proG-CVIApf was added to the wells and the slide was incubated for 2 h at rt. The silicon mat was removed, the slide was washed with PBST buffer three times for 1 h at rt, and the slide was visualized.

\section{RESULTS AND DISCUSSION}

\textbf{PEG-Alkane Spacers and Linkers.} PEG-alkane derivatives were prepared with azide groups attached to the PEG end of the molecules and hydroxyl (spacer), thiol (linker), or amine (linker) groups on the hydrocarbon end (see Scheme 1 for structures). Thiols 1 (spacer) and 2 (linker) were prepared by the general protocol reported by Pale-Grosedemange et al. (Scheme S1).\textsuperscript{31} Spacer 1 has a hydrophobic undecamethylene chain at the thiol end and a triethylene glycol chain at the hydroxyl terminus. The thiol end of linker 2 is similar, while the azide end of the molecule contains a tetaethylene glycol unit. Amine 3 (spacer) has a hexamethylene unit at the amino end and a triethylene glycol chain at the hydroxyl terminus. Amine 4 (linker) has a hexamethylene unit (amine end) attached to a PEG8 azide (azide end) through amide linkages to a diglycycloic acid spacer.\textsuperscript{26,30}

\textbf{Preparation of SAMs on Gold Slides and Modified Proteins.} SAMs were prepared from ethanol solutions of a mixture of the thiol spacer and linker (thiol SAMs) or a mixture of amine spacer, amine linker, and carbon disulfide for dithiocarbamate (DTC) SAMs. The gold slides were imaged before and after SAM formation. N-terminal His\textsubscript{6}-tagged GFP-CVIA and GST-CVIA were expressed and purified as previously described.\textsuperscript{26,29} Incubation of the proteins with FPP (control) or ω-propargylFPP (Figure 1) with PFTase as described by Gauchet et al. gives GFP-CVIAf, GFP-CVIApf, GST-CVIAf, and GST-CVIApf, respectively.\textsuperscript{27} The modifications were confirmed by ESI MS.\textsuperscript{30} Modified GFP retained its native fluorescence properties, and modified GST retained its catalytic activity.\textsuperscript{27} The regio- and chemoselective immobilization of GFP-CVIApf and GST-CVIApf on SAM-coated gold

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{FPP and ω-propargylFPP.}
\end{figure}
slides was carried out as described by Seo et al. for silica surfaces. 30

**Covalent Immobilization on Thiol SAMs.** The thiol SAM technique has been widely used to attach biological molecules, including antibodies and enzymes, to gold surfaces. 6,7 Regioselective covalent immobilization of proteins on SAMs can be accomplished without denaturation, resulting in an increased binding capacity and sufficient exposure of functional domains. The alkanethiol assembly on gold was used for site-specific immobilization of GFP-CVIApf and GST-CVIApf shown in Scheme 1. Part A of Figure 2 shows images of wells where GFP-CVIApf in PBS buffer was attached to SAMs containing different ratios of spacer 1 and linker 2. Lane a is a copper-free control; lane b has CuSO4, TCEP, and TETA; lane c contains CuSO4, and lane d has CuSO4 and TCEP. A 9:1 ratio of 1:2 gave optimal performance (i.e., high sensitivity and low background). Immobilizations with higher concentrations of GFP-CVIApf (20 and 80 μM) on thio-SAMs containing different ratios of spacer 1 and linker 2 were examined, confirming that a 9:1 ratio of 1:2 was optimal (Figure S1). As expected, GFP-CVIApf was not efficiently immobilized in wells without Cu(II) or with CuSO4 and no TCEP reductant, although a small amount of immobilization, presumably non-specific, was observed in wells containing CuSO4 apparently promoted by the presence of Cu(II). This amount increases in the wells containing CuSO4 and TCEP, a phenomenon previously reported by Speers and Cravatt. 32 Under our conditions, Cu2+ does not promote significant nonspecific binding of GFP-CVIApf.

Part B of Figure 2 shows fluorescence intensities for pairs of slides with SAMs consisting of 9:1:spacer 1:linker 2 (left) or only spacer (right). Individual wells contained 1–10 μM GFP-CVIApf and CuSO4/TCEP/TBTA in PBS buffer. The slides coated with spacer 1:linker 2 (9:1) showed a concentration-dependent increase in fluorescence intensity for GFP. Detection of GFP fluorescence established that the native protein remained folded after immobilization. As expected, no fluorescence was observed in control wells without protein. Very weak spots of fluorescence for GFP-CVIApf detected on the spacer-only slides indicated that a small amount of the protein remained on the SAM after washing. Similar patterns were observed after an additional 16 h wash when the slides were imaged at higher sensitivity with fluorescent antiGFP680. Significant levels of fluorescence were observed in those wells treated with 4–10 μM of GFP-CVIApf, indicating that a ~5 μM concentration of the alkyne-modified protein was sufficient for detection.

In a second set of experiments (Figure 3), fluorescence intensity was measured in wells of slides coated with spacer 1:linker 2 (9:1) containing varying concentrations of GFP-CVIApf or GFP-CVIaf. Lane A contained 50 μM CuSO4, 50 μM TCEP, 50 μM TBTA. Lane B: 0.5 mM CuSO4, 0.5 mM TCEP, 50 μM TETA. Fluorescence intensity was measured at λEx = 532/λEm = 526 nm for the detection of GFP and at λEx = 633/λEm = 670 nm for antiGFP680.

**Protein Immobilization on DTC-SAMs.** The most commonly used procedure for preparing SAMs on gold surfaces is by chemisorption of thiols. However, surface-bound thiols chemisorbed by a single sulfur–gold bond can leach from the gold surfaces, and care must be taken during synthesis and storage of thiols to prevent oxidation. Recently, Zhao and co-workers reported formation of SAMs with dithiocarbamate (DTC) linkages to gold surfaces by treatment with a mixture of an amine and carbon disulfide (CS2). 15 The enhanced stability of the DTC-SAMs in acidic and basic conditions was attributed to superior chemisorption properties due to the formation of two sulfur–gold bonds. 33,34

We prepared DTC-SAMs by shaking gold-coated slides in an ethanol solution of CS2 and primary amines, spacer 3 and linker 4, which we previously attached to silica surfaces through urea linkages 30 (Scheme 2). Unlike thiol-SAMs, there was no significant difference in the fluorescence intensities of GFP-
Scheme 2. Preparation of DTC-SAMs on Gold Surfaces and Immobilization of GST-CVIApf by a Huisgen Cycloaddition

CVIApf on SAMs prepared with different molar ratios of spacer 3 and linker 4 (Figure 4A) when measured directly or after incubation with fluorescent antiGFP680. As observed for the thiol SAMs, wells without Cu(I) did not fluoresce (lane a, Figure 4A); those containing CuSO₄ were weakly fluorescent, while those containing CuSO₄ and a mixture of TCEP and TBTA or TCEP alone gave strong signals. These trends were observed for direct visualization of GFP or indirect detection of GFP-CVIApf.

Figure 4. Visualization of GFP-CVIApf immobilized on different molar ratios of spacer 3/linker 4 DTC-SAMs. (A) 5 μM GFP-CVIApf; (a) PBS; (b) 1 mM CuSO₄, 1 mM TCEP, 100 μM TBTA in PBS; (c) 1 mM CuSO₄ in PBS; (d) 1 mM CuSO₄ and 1 mM TCEP in PBS. (B) Varied concentrations of GFP-CVIApf; 0.5 mM CuSO₄ (+) and without CuSO₄ (−), 0.5 mM TCEP and 50 μM TBTA in PBS; slide was coated with a 9:1 molar ratio of spacer 3 and linker 4. Fluorescence intensity was measured at λₐ = 532/λₑm = 526 nm for the detection of GFP and at λₐ = 633/λₑm = 670 nm for antiGFP680.

The experiments described in Figure 3 were repeated for gold slides coated with DTC-SAMs formed from spacer 3 and linker 4 (9:1) with similar results (Figure 5). GFP fluorescence was observed in wells treated with 500 μM CuSO₄, 500 μM TCEP, 50 μM TBTA, and 20–80 μM GFP-CVIApf; and very weak signals were detected in wells treated with 500 μM CuSO₄, 500 μM TCEP, 50 μM TBTA, or those treated with 500 μM CuSO₄, 500 μM TCEP, 50 μM TBTA, and 80 μM GFP-CVIAf. The intensity of the spots increased when visualized with antiGFP680.

The selectivity for detection of immobilized proteins by fluorescent antibodies was determined with a gold slide coated with DTC SAMs. GST-CVIApf and GFP-CVIApf were immobilized in wells on the top and bottom halves of the slide, respectively, washed and imaged. Fluorescence was observed for the GFP fluorophore (Figure 6). The slide was washed again and incubated with a mixture of antiGST488 and antiGFP680. When the slide was imaged, fluorescence was detected at 526 nm (excitation 532 nm) for wells containing GST-CVIApf and at 670 nm (excitation 633 nm) for wells containing GFP-CVIApf. When the slide was imaged at 532/526 nm, fluorescence was detected in wells immobilized with GFP-CVIApf along with wells containing antiGST488. However, fluorescence intensity of GFP-CVIApf at 532/526 nm is weak compared to that of antiGST488, and only antiGST488 was visualized when their fluorescence intensities were measured together.

Quantitation of Immobilized proG-CVIApf on Thiol and DTC-SAM Coated Slides. Different concentrations of proG-CVIApf were spotted into wells of a matted slide coated with thiol- and DTC-SAMs. The mat was removed before the slide was washed and incubated with Alexa680 goat anti-rabbit IgG. Plots of fluorescence intensity as a function of proG-CVIApf concentration showed that the increase in fluorescence reached a plateau when 10 μL of a ~1.6 μM solution of proG-CVIApf (5.1 pmol/mm²) was used for immobilization to the thiol SAM (Figures 7 and S4). For the DTC-SAM surface, the plateau was reached when 10 μL of a ~16 μM proG-CVIApf solution (51 pmol/mm²) was applied.

A surface coated DTC-proG-CVIApf slide was prepared by treatment with 30 μL of a solution containing 100 μM proG-CVIApf (2.3 pmol/mm²) and 30 μL of Cu(I) buffer. A silicon mat was attached and 10 μL samples of a series of concentrations of Alexa488 anti-GST rabbit IgG (MW ~150K) were spotted into the wells. A plot of fluorescence intensity versus concentration indicates that 10 μL of a 20 μg/
μL solution (0.42 pmol/mm²) of the antibody saturates the binding sites of immobilized proG-CVIApf (Figures 8 and S5).

Displacement of Immobilized Proteins in Thiol- and DTC-SAMs by β-Mercaptoethanol. The relative ease of displacing immobilized proteins from thiol and DTC SAMs on gold slides was examined with a solution of β-mecaptoethanol (BME) in ethanol. Slides with GFP-CVIApf immobilized on thiol and DTC-SAMs were incubated in a solution of BME in ethanol and imaged with antiGFP680. The intensity of the fluorescent signals of wells on thiol-SAMs decreased by approximately 94% when incubated in 50 mM BME for 24 h (Figure 9A). A slightly less intense signal was observed after incubation for an additional 24 h in 100 mM BME. After 46 h, the signal had decreased by 99% from its original intensity. In contrast, displacement was much slower for GFP-CVIApf immobilized on DTC SAMs. Under similar conditions, the signal intensity decreased by only 13% after 48 h and 27% after 72 h (Figure 9B). It is evident that the sulfur−gold linkages in DTC-SAMs are substantially more robust than those in thiol SAMs.

CONCLUSION

There are many different approaches for immobilizing proteins to create biochips. The technique described here involving SAM-coated gold surfaces provides a relatively simple general procedure to covalently immobilize proteins regio- and chemoselectively using a bioorthogonal Huisgen [3 + 2] cycloaddition. The immobilized proteins, in this work, GFP, GST, and proG, were immobilized directly from cell-free homogenates chemoselectively and visualized with fluorescent antibodies. While the proteins can be immobilized on either thiol- or DTC-coated gold surfaces, DTC SAMs are more stable and amine-terminated linkers used to construct the SAMs are easier to handle and store than their thiol-terminated counterparts.

Figure 6. Selective detection of GST and GFP with fluorescent antibodies. GST-CVIApf and GFP-CVIApf (80 μM) were immobilized on the same slide (1:9 spacer linker 4 DTC-SAMs), and then incubated with a mixture of antiGST488 (2 μg/mL) and antiGFP680 (2 μg/mL). Well treated with (+) and without (−) Cu⁺. Fluorescence intensity was measured at λₐₑₓ = 532/λₐₑₘ = 526 nm for detection of antiGST and at λₐₑₓ = 633/λₐₑₘ = 670 nm for antiGFP.

Figure 7. Plots of fluorescence intensities vs varied concentrations of protein G. ProG-CVIApf was immobilized on 9:1 molar ratio of spacer/linker (A) thiol-SAMs or (B) DTC-SAMs by Cu(I) catalyzed cycloaddition (0.5 mM CuSO₄, 0.5 mM TCEP, 0.05 mM TBTA). The slides were incubated with Alexa 680 labeled goat anti-rabbit IgG (1 μg/mL). Well treated with (+) and without (−) Cu⁺. Fluorescence intensity was measured at λₐₑₓ = 633/λₐₑₘ = 670 nm for the detection.

Figure 8. Plot of fluorescence intensity vs varied concentration of anti-GST rabbit IgG. ProG-CVIApf (50 μM) was immobilized on a slide with 9:1 molar ratio of spacer/linker DTC-SAM by Cu(I) catalyzed cycloaddition (0.5 mM CuSO₄, 0.5 mM TCEP, 0.05 mM TBTA). A series of 10 μL samples of different concentrations of anti-GST rabbit IgG (Alexa Fluor 488 conjugate) were spotted in the wells. Fluorescence intensity was measured at λₐₑₓ = 532/λₐₑₘ = 526 nm.

Figure 9A. A slightly less intense signal was observed after incubation for an additional 24 h in 100 mM BME. After 46 h, the signal had decreased by 99% from its original intensity. In contrast, displacement was much slower for GFP-CVIApf immobilized on DTC SAMs. Under similar conditions, the signal intensity decreased by only 13% after 48 h and 27% after 72 h (Figure 9B). It is evident that the sulfur−gold linkages in DTC-SAMs are substantially more robust than those in thiol SAMs.
linkers. When used to immobilize antibody-binding proteins, this technique can be extended to detect a wide variety of molecules.30

ASSOCIATED CONTENT
Supporting Information
NMR and MS related to the synthesis are available. 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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