Published in final edited form as:

Langmuir. 2011 February 15; 27(4): 1463–1471. doi:10.1021/la104186n.

Spatially Addressable Chemoselective C-terminal Ligation of an Intein Fusion Protein from a Complex Mixture to a Hydrazine-Terminated Surface

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Abstract

Protein immobilization on surfaces is useful in many areas of research, including biological characterization, antibody purification, and clinical diagnostics. A critical limitation in the development of protein microarrays and heterogeneous protein-based assays is the enormous work and associated costs in the purification of proteins prior to their immobilization on a surface; methods to address this problem would simplify the development of interfacial diagnostics that use a protein as the recognition element. Herein, we describe an approach for the facile, site-specific immobilization of proteins on a surface without any preprocessing or sample purification steps that ligates an intein fusion protein at its C-terminus by reaction with a hydrazine group presented by a surface. Furthermore, we demonstrate that this methodology can directly immobilize a protein directly from cell lysate on to a protein-resistant surface. This methodology is also compatible with soft lithography and inkjet printing, so that one or more proteins can be patterned on a surface without need for purification.

Keywords

Protein; patterning; intein; site-specific immobilization; microarray; cell lysate

Introduction

Protein immobilization and patterning¹ on surfaces is important for a range of applications that span affinity chromatography,² biosensors,³ heterogeneous immunoassays,⁴ and protein microarrays.⁵ Although many different methods of protein immobilization have been developed,⁶ there are few, if any methods that address all of the following critical needs: (1) the method must be efficient, with high coupling yield of the protein to the surface; (2) it should be generically applicable to most proteins with no post-expression modification of the protein; (3) it should be site-selective, so that the protein is attached at one site to the surface with a uniform orientation; (4) it must be compatible with common methods to spatially address one or more proteins on the same surface; and (5) it should be

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bioorthogonal⁷, so that the protein of interest can be covalently ligated to the surface from a complex mixture.

Herein, we describe a method to immobilize and pattern proteins on a surface that fulfills these requirements. This method involves the site-specific, C-terminal immobilization of recombinant proteins on a surface in which the reactants –the C-terminal reactive site of the protein and the functional group presented by the surface– are highly orthogonal to other functional groups present in biological mixtures, so that it can be carried out with high selectivity and sensitivity from a complex mixture such as cell lysate (Scheme 1). This approach has two components: a recombinant fusion of a target protein with an intein⁸–a self-splicing protein domain– and a surface that presents a hydrazine moiety. The fusion of an intein at the C-terminus of a protein creates a peptide bond between them, which spontaneously forms a dynamic equilibrium with the thioester bond, resulting in an N->S shift intermediate product. This highly reactive thioester bond can then be attacked by a reactive nucleophile, such as hydrazine, as originally demonstrated by Raines and coworkers for the post-expression azide modification of a protein.⁹

We exploited this reaction to show that incubation of an intein fusion protein with a hydrazine moiety that is immobilized on a protein-resistant surface [Figure S1 in Supporting Information (SI)] results in the surface-mediated cleavage of the intein from the target protein, resulting in the site-specific ligation of the C-terminus of the protein on the substrate in a one-step process (Scheme 2A, left) without prior cleavage of the intein from the target protein. We also compared the hydrazine-intein conjugation methodology with two other, more established methods. The first method is intein-mediated expressed protein ligation (EPL) (Scheme 2A, right) that uses the same general principle except that it is a two-step process which requires cleavage of the intein from the target protein to liberate the thioester and a separate ligation step with a cysteine-functionalized surface, in contrast to the surface-triggered, one-step intein-mediated ligation that forms the core of this paper (Figure S2 in SI). Although Xu *et al* have reported that cysteine-containing molecules could also directly attack the thioester bond in an intein domain,^{10a} this reaction has not been demonstrated on a surface, so that current EPL on surfaces is typically carried out as a two-step process. The second method that we compared with the surface-triggered intein ligation is EDC/NHS chemistry, which results in the attachment of the protein via solvent accessible amine groups on the protein surface with the EDC/NHS-activated carboxyl groups on the surface. (Scheme 2B, left). We also demonstrate that the surface-mediated intein ligation methodology is compatible with soft lithography and inkjet printing, so that one or more proteins can be patterned on a surface without need for purification.

Experimental Section

Surface modification steps to fabricate hydrazine and cysteine surfaces

Carboxy-hexa(ethylene glycol) undecanethiol (EG6-COOH) and tri(ethylene glycol) undecanethiol (EG3-OH) were purchased from Prochimia. For fabrication of the hydrazine-derivatized SAM, a pure EG6-COOH or a mixed EG6-COOH/EG3-OH surface was incubated in an EDC (0.1 M)/NHS (0.2 M) solution in water for 15 min to activate the -COOH groups. Next, the SAM was sequentially incubated in the following solutions at room temperature (RT) for 2 h: (1) 1 M alkyne-amine (propargylamine) (Figure S1 in SI) in THF; (2) 1 M 2-(2-aminoethoxy) ethanol (AEE) in HEPES buffer (50mM, pH 7.4). Finally, the substrate was immersed in a 100 mM azide-hydrazine (Figure S3 in SI) solution in EDTA-free MOPS-NaOH buffer (0.5 M) at pH 7.4, with 0.5 mM copper sulfate and 7.5 mM sodium ascorbate added, and incubated overnight. The modification steps to install the cysteine group on the SAMs were similar to those used for hydrazine derivatization, including: 1) EDC /NHS activation of the -COOH groups in mixed SAMs; 2) sequential

incubation in the following solutions at RT for 2 h: (a) 1 M azide-amine (11-azido-3,6,9-trioxaundecan-1-amine) (Figure S2 in SI) in HEPES buffer (50mM, pH 7.4); and (b) 1 M AEE in HEPES buffer (50 mM, pH 7.4). Finally, the substrate was immersed in a 50 mM alkyne-cysteine (Figure S3 in SI) solution in EDTA-free PBS (pH 7.4), with 0.5 mM copper sulfate and 7.5 mM sodium ascorbate added, and incubated overnight. Freshly prepared, functionalized chips were used in all protein binding experiments.

Protein immobilization

All fusion proteins were expressed in *E. coli*,¹¹ and the concentration was determined by UV-vis spectroscopy. *E. coli* thioredoxin (Trx) for NHS/EDC coupling was from Sigma with a minimum purity of 90% (SDS-PAGE). For the hydrazine surface, purified thioredoxin (Trx)-intein-fusion protein was reacted directly with the surface. For the cysteine surface, Trx was pre-cleaved from the Trx-intein-fusion by adding either dithiothreitol (DTT) or 4-mercaptophenylacetic acid (MPAA) to the protein solution. For Surface Plasmon Resonance (SPR), protein solutions in HBS-EP buffer (pH 7.4, TCEP 0.5 mM) were injected into the flow cell at a flow rate of 1 μ l/min, in a total injection volume of 100 μ l. An *E. coli* Anti-Trx monoclonal antibody (mAb) was then injected to bind to the protein-ligated surface to evaluate antibody recognition. For binding to micropatterns, gold substrates were first patterned with EG6-COOH thiol by microcontact printing (μ CP), followed by incubation in a solution of EG3-OH thiol to render the background regions resistant to the adsorption of proteins. The patterned, functionalized surface was then sequentially incubated in the target protein solution of interest and anti-Trx mAb (0.01 mg/ml), for 2 h at RT, and then incubated in a secondary, fluorescently labeled, antibody solution (Alexa 647-IgG) (0.01 mg/ml) at 37°C for 2 h. For protein printing, protein samples in printing buffer at different concentrations were spotted on a hydrazine-modified, gold coated, coverslip using a contact microarrayer (AxSys MFC, Cartesian Tech) at room temperature and 80% humidity, with 10% to 50% glycerol added to the spotting sample buffer, and incubated under these conditions for 40 h. Green Fluorescent Protein (GFP) spots were directly observed under fluorescence microscopy after rinsing, and Trx spots were further incubated in anti-Trx mAb (0.01 mg/ml) for 2 h at RT, and then a secondary, fluorescently-labeled, antibody solution (Alexa 647-IgG) (0.005 mg/ml) at 37°C for 2 h. Thorough rinsing in HBS-EP buffer (three cycles with each cycle consisting of at least 6 min continuous shaking) was used between each step in the above static binding, micropatterning, and printing experiments.

Characterization

Protein concentrations were calculated from their UV-vis spectra (Nanodrop). Surface Plasmon Resonance (SPR) was performed on a Biacore X spectrometer (Biacore, GE). X-ray photoelectron spectra (XPS) were obtained on an AXIS Ultra (Kratos Analytical, NY) spectrometer with a monochromatic AlK α X-ray source (1.4867 KeV) and a monochromator. Operational conditions for the X-ray source were 400 μ m nominal X-ray spot size operating at 15 kV, 8.9 mA for both survey and high-resolution spectra. Survey spectra, from 0 to 1200 eV binding energy (BE), were acquired at 100 eV pass energy with a step size of 1.0 eV, and a dwell time of 100 ms. High-resolution spectra were recorded at 20 eV pass energy with an energy step of 0.1 eV, a dwell time of 1.2 s, with a typical average of 50 scans. XPS measurements on a pure EG6-SAM functionalized sample showed that the experimental C/O ratio was very close to the theoretical C/O ratio, suggesting that carbon contamination was neglectable. Fluorescence microscopy was performed with a Nikon Eclipse TE2000-U. The protein-immobilized surface was kept hydrated during fluorescence microscopy to prevent denaturation of the protein. For GFP observation, a B function filter set was used with the excitation wavelength at 488 nm and the emission wavelength at 509

nm. For Trx observation after labeling with Alexa 647-IgG, a Cy5 filter set was used with the excitation wavelength at 649 nm and the emission wavelength at 666 nm.

Results

Expression and purification of Trx-intein fusion protein

To demonstrate proof-of-principle of this approach, a Trx-intein fusion was synthesized in *E. coli* from a plasmid-borne gene, in which Trx was fused at its C-terminus to the N-terminus of an intein. We used Trx as the model protein for most of these studies as it is soluble, and expresses with high yield in *E. coli* as an intein fusion.¹¹ For arraying experiments, we also used a GFP-intein fusion to demonstrate the arraying of more than one protein on the same substrate by contact printing. The C-terminus of Trx and GFP were fused with the N-terminus of a mutant mini-intein, encoded by the *Mycobacterium xenopi* GyrA gene (Mxe).^{10b} A purification tag, an elastin-like polypeptide (ELP), was fused to the C-terminus of the intein at the gene level,¹² to enable easy purification of the tripartite fusion protein by a simple non-chromatographic purification method we have developed, inverse transition cycling (ITC), for experiments that required purified protein.¹³ Although the ELP tag was used in this work for experiments requiring purified samples, the ELP is not necessary, as the Trx-intein fusion can be ligated to a hydrazine-functionalized surface directly from crude cell lysate, as we show in this paper.

The recombinant synthesis, expression, and purification of this tripartite Trx-intein-ELP fusion protein has been described elsewhere,¹⁰ and is hence not reported here in detail. Briefly, however, both the Trx-intein-ELP fusion and the GFP-intein-ELP fusion proteins were expressed in *E. coli* and purified by ITC. The purified Trx-intein-ELP (and GFP-intein-ELP) were characterized by SDS-PAGE; results for Trx-intein-ELP are shown in Figure 1A. Two bands were observed for the purified protein; the top band at ~72 kDa corresponds to the Trx-intein-ELP fusion while the second band at ~60 kDa corresponds to the premature intracellular cleavage product, intein-ELP, which occurs in the reductive expression intracellular environment of *E. coli*.¹⁰ The fraction of the intact Trx-intein-ELP in the purified protein was estimated to be ~60%. The free Trx that was cleaved intracellularly from the intein-ELP did not appear in the gel because it did not contain an ELP tag and was therefore lost during ITC purification. No other contaminating protein bands were observed. We chose not to purify the two bands further to separate the parent Trx-ELP-intein fusion from the cleavage product (intein-ELP) because the latter protein cannot attach to the surface by either intein-mediated ligation reaction.

SAM formation

We chose SAMs of oligoethylene glycol-functionalized alkanethiols (OEG-SAMs) on gold as the surface for these experiments for the following reasons: alkanethiol SAMs are: (1) easy to reproducibly prepare; (2) easy to characterize by a range of spectroscopic techniques; (3) yield well-defined organic interfaces; (4) at which a functional group of interest—herein, a hydrazine or COOH—can be presented with controlled surface density via formation of a mixed SAM of two different alkanethiols, only one of which contains the terminal functional group of interest; (5) on a protein-resistant oligoethylene glycol background; the protein resistance of these SAMs is critical to the successful implementation of our methodology from crude cell lysate. This last attribute of OEG-SAMs, while critical to the success of patterning from cell lysate, does not, however limit the methodology to SAMs on gold, as other protein resistant surfaces, such as spin-cast films of a co-polymer of oligoethylene glycol methacrylate (OEGMA) and methyl methacrylate¹⁴ and polymer brushes of OEGMA¹⁵ provide alternatives that will allow this methodology to be used with other materials of interest, such as glass¹⁶ and plastics¹⁷.

In order to quantitatively compare the three methods, chemical reaction routes were designed to obtain a similar density of the functional groups that react with the protein in each method (Scheme 2B). The detailed surface reaction routes to obtain the three different functionalized interfaces are shown in Supporting Information (Figures S1 and S2 in SI). The initial step in the fabrication of reactive interfaces for all three protein immobilization methods was the self-assembly of a mixed SAM of EG6-COOH and EG3-OH on a gold surface. We chose SAMs on gold that present either a hexaethylene glycol or a triethylene glycol moiety at their termini because of the ability of oligoethylene glycol-terminated alkanethiol SAMs to resist the adsorption of proteins,¹⁸ a feature that was critical to our effort to spatially address proteins on a surface from crude *E. coli* lysate. To couple Trx-intein-ELP to the surface by surface-triggered intein ligation, an amine-terminated alkyne was coupled to the -COOH groups presented by the mixed SAM by EDC/NHS chemistry to introduce an alkyne group onto the surface with 86% yield, as determined by the atomic N/C ratio measured by XPS (Table S1 in SI). The alkyne group was then reacted with an azide-terminated hydrazine (Figure S3 in SI) using click chemistry with 73% yield, also determined by XPS (Figure S4 and Table S1 in SI). The unreacted COOH was quenched by AEE.^{19, 20} The detailed reaction route is shown in SI (Figure S1 in SI), and the total conversion yield of carboxylic acid groups to hydrazine groups on the surface was ~63% ($86\% \times 73\%$).

For coupling the Trx-intein fusion by conventional EPL, it was necessary to install a cysteine residue at the surface. To do so, an amine-terminated azide derivative was coupled to the carboxylic acid groups on the surface via EDC/NHS (63% yield,²¹ Table S1 in SI), after which an alkyne-terminated cysteine (Figure S3 in SI) was reacted with the azide-functionalized surface using click chemistry (78% yield, Figure S5 and Table S1 in SI). Similarly, the unreacted COOH was quenched by AEE.^{19, 20} The detailed reaction scheme is shown in SI (Figure S2 in SI). The composite yield of the conversion of carboxylic acid to cysteine was 50% ($63\% \times 78\%$). For coupling Trx using EDC/NHS chemistry, no modification of the COOH-terminated SAM was necessary. We did not verify the degree of conversion of the -COOH groups to the reactive, NHS ester because of the sensitivity of these groups to hydrolysis, but a substantial body of evidence indicates that a yield of 50–80% is routinely obtained on SAMs on gold.¹⁹ These results, therefore, suggest that the three surfaces had a similar concentration of the relevant reactive functional groups, which allowed quantitative comparison of the immobilization of Trx on these surfaces by the three methods.

Surface-triggered intein protein ligation

We first investigated the covalent attachment of the Trx-intein-ELP fusion protein to a 50% hydrazine surface using SPR (Figure 1B). Trx-intein-ELP was injected at a concentration of 0.01 mg/ml in buffer over the hydrazine surface, and then rinsed to remove any non-specifically bound protein. An increase in the SPR signal of 1200 RU indicated substantial protein binding to the surface. In order to determine whether the Trx was attached to the surface in an orientation such that the binding sites were accessible and active, an anti-Trx mAb (0.1 mg/ml) was flowed over the surface. After a buffer wash to remove non-specifically bound mAb, an increase in the SPR signal of 1000 RU was observed, indicating significant antibody binding to the immobilized Trx on the surface. In control SPR experiments, in which Trx-intein-ELP was replaced with Trx-ELP (no intein) or with Trx-intein-ELP cleaved with DTT²² prior to injection, no binding of the protein to the surface was measured by SPR (Figure S6 in SI), suggesting that the hydrazine-terminated SAM exhibits selective reactivity towards the peptide bond that connects Trx to the intein in the Trx-intein-ELP fusion protein. The effect of the surface density of the hydrazine functional group on protein immobilization was also investigated by SPR (Figure 1C). The increase in

SPR signal upon Trx binding to hydrazine at the surface was dependent on the surface density of hydrazine (10%, 25%, 50%, and 100%), and a greater signal increase was observed corresponding to a higher surface density of hydrazine groups. We did not observe a significant difference in the SPR signal between the 50% and 100% hydrazine surfaces, suggesting that the surface was already saturated with bound protein at the lower hydrazine surface density.

Comparison with EPL and EDC/NHS coupling

Having established the feasibility of surface-triggered intein ligation on the mixed SAMs, we compared it with conventional EPL and EDC/NHS coupling. For conventional EPL, Trx was first cleaved from the Trx-intein-ELP fusion protein by the addition of free thiol in solution to yield a thioester-terminated Trx (Figure S7 in SI), which could then react with a cysteine-functionalized SAM via EPL. The reactivity of the resulting thioester strongly depends on the type of thiol used to cleave intein fusion proteins, as Kent et al. showed that the use of a benzyl thiol reagent like MPAA increased the efficiency of EPL in solution compared to an alkyl thiol.²³ We hence used both DTT and MPAA to compare the efficiency of Trx coupling to a cysteine-terminated SAM to that obtained for the hydrazine-terminated SAM.

The site-specific attachment of the Trx-thioester liberated by cleavage with DTT or MPAA to a 50% cysteine-functionalized surface by conventional EPL was investigated by SPR (Figure 2A). In initial scouting experiments, we attempted to immobilize Trx, liberated from the Trx-intein-ELP fusion with DTT or MPAA, at the same Trx concentration of 0.01 mg/ml that was used for immobilization of the Trx-intein-ELP to a hydrazine-terminated SAM. Minimal attachment to the surface was observed at 0.01 mg/ml by SPR (Figure S8A in SI). We hence used a 10-fold greater concentration of 0.1 mg/ml for the attachment of Trx-intein-ELP fusion to the cysteine-functionalized SAM. A protein concentration of 0.1 mg/ml resulted in an increase in signal of 400 RU for Trx cleaved with MPAA from the intein fusion as compared to the increase of 200 RU observed for the DTT-cleaved intein fusion, which is consistent with the findings of Kent et al.²³ Further increase of the Trx-thioester concentration to 1 mg/ml did not yield results that were significantly different from those at 0.1 mg/ml. Because the surface density of cysteine residues is similar to that of the hydrazine-modified surface, these results indicate that the level of attachment by conventional EPL was significantly lower than the hydrazine-mediated strategy, even at a 10-fold greater concentration of protein (Figure 2C). Control experiments using commercially available Trx without a thioester modification resulted in no attachment to a cysteine-functionalized surface (Figure S8B in SI), suggesting that the cysteine-terminated SAM was selectively reactive towards the thioester functional group of DTT or MPAA-based Trx products.²⁴

In order to demonstrate the advantages of surface-triggered intein ligation, EDC/NHS coupling was also used to immobilize Trx on an EG6-COOH SAM. SPR measurements of Trx attachment and subsequent anti-Trx mAb binding on 50% EG6-COOH SAM on gold are shown in Figure 2B. The COOH groups were first activated by EDC/NHS and then reacted with Trx (1 mg/ml). To quench any unreacted sites, the surface was treated with AEE before injection of anti-Trx mAb. Trx attachment to the surface resulted in a SPR signal increase of 1150 RU. These experiments suggested that the level of attachment of the protein was similar to that obtained for the hydrazine-mediated intein ligation (Figure 2C) but only at a ~100-fold greater protein concentration, as lower concentrations of 0.01 mg/ml or 0.1 mg/ml of Trx resulted in no binding of the protein to the 50% COOH-terminated SAM (Figure S9 in SI). These results clearly demonstrate the significantly greater reactivity of hydrazine-mediated surface-triggered ligation compared to the conventional EPL and EDC/NHS coupling even at 10- to 100-fold lower protein concentrations.

We speculated that another potential advantage of site-specific ligation via hydrazine-mediated intein ligation as compared to EDC/NHS coupling would be the uniform orientation of the immobilized protein, which depending on the protein, could translate to a greater accessibility of the active site. We hence used antibody recognition of the immobilized protein to test this hypothesis. A similar evaluation method has been also used by Whitesides et al to assess the orientation of proteins immobilized on surface by a His-tag.²⁵ The results for Trx immobilization by the three different methods and subsequent antibody binding to the Trx-decorated surfaces for three independent replicates ($n = 3$) are shown in Figure 2C. Antibody binding to Trx immobilized on the EG6-COOH SAM by EDC/NHS resulted in a signal of only 200 ± 80 RU, even though Trx immobilization was relatively high (1150 ± 100 RU) and similar to the 1200 ± 100 RU obtained for Trx immobilization on the hydrazine-terminated surface. Despite these similar levels of Trx immobilization for the two methods, antibody binding to Trx immobilized on the hydrazine surface resulted in a five-fold greater signal of 1116 ± 100 RU (Figure 2C). These results clearly indicate that the hydrazine-mediated surface ligation provides a more uniform protein orientation, which in this instance translates to better protein-antibody recognition. In contrast, conventional EPL with MPAA, as previously noted, showed a three-fold lower attachment of the protein with an increase in signal of only 400 ± 100 RU, with an absolute level of attachment of the anti-Trx mAb of 452 ± 50 RU (Figure S10 in SI). Comparison of the optical mass ratios for Trx immobilization and anti-Trx mAb binding for the three methods (Figure 2D) shows that both hydrazine-mediated intein ligation and conventional EPL led to a similar ratio of antibody to protein on the surface, with the only distinction between these approaches being the three-fold greater concentration of protein that was attached to the surface by the hydrazine-mediated approach than conventional EPL with MPAA at a 10-fold lower protein concentration. In contrast, the antibody to protein ratio of 0.93 for the hydrazine-intein method was five-fold higher than the ratio of 0.17 for the EDC/NHS method, although the total amount of Trx coupled to the surface was similar to that obtained for the hydrazine-mediated ligation of Trx-intein-ELP. A likely explanation of these results is that EDC/NHS coupling led to a range of Trx orientations on the surface, so that the mAb binding epitope in a large fraction of the immobilized Trx was not accessible to the antibody, thereby leading to a low amount of mAb binding to the surface.

Micropatterning by Microcontact Printing (μ CP)

We next examined whether the Trx-intein-ELP fusion protein could be directly immobilized on a hydrazine-patterned SAM directly from pure solutions and from cell lysate. This experiment was motivated by the following rationale: first, micropatterning provides an internal control on the fidelity and selectivity of the immobilization methodology of the process by visualization and quantification of the S/N between the pattern and background. Second, the micro- and nano-patterning of bioactive proteins to direct cell spreading, growth, and differentiation in a spatially controlled fashion has become an important tool in cell biology,²⁶ biomaterials,²⁷ and tissue engineering,²⁸ so that a methodology to pattern recombinant proteins from cells that express the protein without the tedious and time-consuming intermediate steps of purification would provide a useful tool to researchers in these fields.

We fabricated protein micropatterns by μ CP the EG6-COOH thiol, and then backfilled the background by formation of a SAM of the EG3-OH alkanethiol. Subsequent steps of modification of the -COOH group to present a hydrazine group, a cysteine residue, or an NHS ester proceeded as described previously for coupling to unpatterned surfaces by the three immobilization methods. The images in Figure 3A correspond to patterns fabricated by the following methods: surface-triggered intein ligation on a patterned hydrazine-terminated EG6-thiol (Figure 3A); EPL on a patterned cysteine-derivatized EG6-thiol (top inset in

Figure 3A); and EDC/NHS attachment on a patterned COOH-terminated EG6- thiol (bottom inset in Figure 3A). As previously discussed, the functional group concentration of the three relevant coupling groups –hydrazine, cysteine, and NHS ester groups– was similar for all three surfaces, which enables direct comparison of the three patterning strategies. After immobilization of the protein (1 mg/ml), the EDC/NHS activated surface was quenched with AEE, and then all three surfaces were incubated with an anti-Trx mAb followed by an Alexa 647-IgG conjugate for visualization by fluorescence microscopy. After thorough rinsing with HBS-EP buffer to remove non-specifically bound protein, the surfaces were characterized by fluorescence microscopy in buffer. All images were acquired under identical conditions, and the contrast in these images shows that the hydrazine-mediated intein ligation methodology resulted in significantly greater contrast between patterned regions and background than the other two methods. The quantified fluorescence intensity data (Figure 3B) confirmed this observation; there was a four- to six-fold greater S/N between pattern and background for Trx patterned by hydrazine-mediated, surface-triggered intein ligation as compared to conventional EPL or EDC/NHS coupling, likely due to the greater surface density of the immobilized protein, as well as its uniform orientation that presented a higher areal density of binding epitopes on the protein to the anti-Trx mAb. In control experiments, when Trx-intein-ELP was replaced with Trx-ELP (no intein) or Trx-intein-ELP that was cleaved with DTT prior to incubation with the hydrazine-patterned surface, no obvious patterns were observed for surfaces that were patterned with hydrazine moieties by μ CP, indicating that the hydrazine surface shows spatially selective reactivity towards the Trx-intein-ELP fusion protein (Figure S11 in SI).

We next examined whether the Trx-intein-ELP fusion protein could be directly immobilized on a hydrazine-patterned SAM directly from cell lysate. To do so, crude cell lysate obtained from BLR *E. coli* cells was spiked with different concentrations of the Trx-intein-ELP fusion protein that ranged from 0.001–0.1 mg/ml. Micropatterned hydrazine-terminated SAMs were then incubated in these solutions to immobilize the Trx-intein-ELP fusion, followed by sequential incubation with an anti-Trx mAb, and an Alexa647-IgG conjugate for visualization by fluorescence microscopy. Bright patterns with good contrast between the hydrazine-patterned regions and the background were observed for fusion protein concentrations of 0.1 mg/ml (Figure 3C) and 0.01 mg/ml (Figure S12 in SI), with patterns still clearly visible for the surface that was incubated with the fusion protein at the most dilute concentration of 0.001mg/ml (10 nM, inset in Figure 3C).

Figure 3D quantitatively summarizes the effect of fusion protein concentration on the S/N between pattern and background from protein patterns that were fabricated from buffer and cell lysate. For the hydrazine patterned SAM that was incubated with purified Trx-intein-ELP fusion protein in buffer, a S/N between pattern and background of ≥ 6 was obtained even at the relatively dilute protein concentration of 0.001 mg/ml. In contrast, patterning from crude cell lysate resulted in a lower S/N at the same protein concentration relative to pure protein, but a S/N between pattern and background of ≥ 2 was obtained at a protein concentration of 0.001 mg/ml. Therefore, these results show that a hydrazine-patterned surface could effectively capture an intein fusion protein even at low nanomolar concentration. In contrast, when crude cell lysate without any Trx-intein-ELP was used as a control, no pattern could be obtained (Figure S13 in SI), indicating that the hydrazine surface could selectively capture target protein from a crude solution containing many other competing and interfering molecules.

We further demonstrated the compatibility of the hydrazine-mediated ligation with common methods to spatially address one or more proteins on the same surface by spotting protein samples on a hydrazine surface using a microarrayer. In Figure 4A, fluorescence images of GFP-ELP and GFP-intein-ELP, printed on a hydrazine-terminated SAM, are shown (panels

I and II, respectively), with both spots having a similar intensity prior to rinsing the surface. After rinsing, the spot corresponding to GFP-ELP (no intein) was washed away (panel III), indicating that the GFP-ELP did not adsorb to the surface, while the spot corresponding to GFP-intein-ELP was still visible (panel IV), indicating chemoselective coupling of the target protein to the hydrazine-functionalized surface. In Figure 4B, Trx-ELP (no intein, panel I) and Trx-intein-ELP (panel II) were printed on the surface, then incubated with anti-Trx mAb and Alexa 647-IgG conjugate to enable visualization of the protein by fluorescence microscopy. The Trx-ELP spots were washed away, indicating no binding, while the Trx-intein-ELP spots remained visible, indicating that the hydrazine-functionalized surface was able to selectively couple the target protein. These results suggested the potential of this method to directly print an intein fusion protein from a complex mixture, and Figure 4C illustrates this potential, showing that a hydrazine-functionalized surface could immobilize both GFP- and Trx-intein fusion proteins directly from cell lysate onto a surface by contact mode printing (5 mg/ml, Figure 4C, panels I and II, respectively) with a lower limit of 0.5 mg/ml intein-fusion in cell lysate (Figure S14 in SI).

Discussion

Traditional methods for immobilizing proteins onto surfaces typically rely on physical adsorption or their covalent attachment via amines or carboxylic acids of the protein.⁶ Unfortunately these methods have significant limitations: (1) they cannot selectively attach a protein of interest from a complex mixture; (2) physical adsorption leads to a variety of protein orientations and conformations on the surface,⁵ while the reactive side-chains of residues that are typically used to attach a protein on a surface such as lysine and cysteine are promiscuously distributed on the surface of most proteins, so that proteins are consequently covalently coupled to a surface in a variety of orientations.⁶

To address these limitations, site-specific immobilization techniques²⁹ have recently emerged. These can be divided into two major classes. The first class uses non-covalent molecular recognition between a capture ligand that is immobilized on a surface and a protein. For example, Dubey et al. recently developed a new self-selective strategy using affinity tags to prepare protein patterns from a solution mixture of two proteins in parallel and with high resolution.³⁰ Similarly Maynard et al employed a multi-step procedure to pattern streptavidin with poly(ethylene glycol) in the background, unpatterned regions for the immobilization of biotin-labeled proteins, through the combination of photolithography and oxime chemistry.³¹ Noncovalent site-specific attachment methods such as the use of an affinity tag³² or DNA hybridization³³ however can encounter stability problems due to dissociation of the noncovalent link between the protein and surface over time.

The second set of methods loosely classified as “bioorthogonal” chemoselective methods³⁴ offer high selectivity by introducing complementary groups on the protein and surface that are designed to covalently react with each other, even in complex biological fluids. Some promising chemoselective immobilization methods have been reported including thiol-ene,³⁵ EPL,³⁶ click chemistry,³⁷ Staudinger ligation,³⁸ Diels-Alder addition,³⁹ intein-based splicing,⁴⁰ and enzyme-catalyzed ligation⁴¹. One of the main drawbacks of some these methods such as EPL and enzyme catalyzed ligation is the low efficiency of the immobilization process,^{34, 42} while methods based on click chemistry and Diels-Alder addition require a bio-orthogonal reactive group to be installed on the protein. The installation of these bioorthogonal reactive groups is typically performed after expression, which adds complexity to the methodology.

To our knowledge, there are only two other conceptually distinct approaches to covalently ligate proteins to a surface from a complex mixture that do not require purification of the

target protein; the first involves the use of protein domains that have high selectivity for a specific ligand that they react with. Examples include thiol-ene reaction between thiol-modified surface and farnesylated proteins,^{35b} intein-based splicing,⁴⁰ EPL,^{32, 36c} the HalotagTM technology⁴³ in which a bacterial haloalkane dehalogenase, fused to a protein of interest, could selectively form a covalent bond with a chloroalkane moiety presented by a solid surface, the cutinase domain that involves the nucleophilic attack of a catalytic serine residue (Ser 120) in the cutinase by an electrophilic phosphonate ligand presented by a surface,⁴⁴ and the O⁶-alkylguanine-DNA alkyltransferase (AGT) domain that selectively reacts with an O⁶-benzylguanine derivative presented by a solid surface.⁴⁵ The second approach involves the direct installation of click groups by site-specific unnatural amino acid incorporation⁴⁶ during expression of the protein of interest.⁴⁷ Both of these methods are valuable, but have their own limitations, such as the large size of the domains in the Halotag, cutinase or AGT technologies that are also attached to the surface, and the complexity and low yield of expression of proteins with site-specific incorporation of unnatural amino acids.

Herein, we have demonstrated a complementary methodology for site-specific C-terminal immobilization of a recombinant intein fusion protein onto a solid surface. We showed that the hydrazine functional groups presented at the terminus of an oligoethylene glycol-functionalized SAM were able to directly attack the peptide bond between the target protein –Trx– and the intein, liberating Trx from the tripartite fusion and covalently attaching it to the SAM on gold. Unlike existing techniques such as EPL, thiol-ene, Halotag, cutinase or AGT immobilization methodologies, the intein domain is not immobilized to the surface, as its only role is to allow the formation of a C-terminal thioester moiety on the target protein. Because the only requirement at the protein level is that the expressed proteins can be expressed as an intein fusion protein, which is readily achieved by recombinant expression of the protein in a heterologous expression system, no post-expression modification of the protein is necessary prior to immobilization, which is usually required in existing bioorthogonal methods.

We also compared this method with two other methods to immobilize proteins – conventional EPL and EDC/NHS coupling– and demonstrated that this methodology has a ~100 fold greater reactivity, as determined by the lowest protein concentration that was efficiently immobilized, compared to conventional intein-mediated EPL on surfaces in a reaction time of 2 h or less.^{36a} Therefore, surface triggered hydrazine-intein methodology overcomes the major limitations –low reactivity and slow reaction kinetics– of conventional intein-mediated EPL on a surface. This new method also provides a higher surface density of the protein as compared to both EPL and EDC/NHS coupling, and better control over protein orientation over EDC/NHS coupling.

We further demonstrated that surface-triggered intein ligation allows proteins to be micropatterned using soft lithography both from a pure, single component solution and from cell lysate at a protein concentration as low as ~10 nM, which corresponds to an expression level of 14.4 µg/L, assuming no concentration of the cells prior to lysis. Moreover, we demonstrated that this technology is also compatible with the contact-mode “quill” printing of proteins from cell lysate to fabricate protein microarrays.

Finally, we note that because the intein fusion protein technology is widely applicable to many proteins that can be solubly expressed in many different expression systems, such as *E. coli*,⁴⁸ yeast,⁴⁹ and mammalian cells,⁵⁰ this technology should be widely applicable to micro- and potentially nanopattern “bioactive” proteins such as growth factors and cytokines that are of interest to the biomaterials and tissue engineering community.⁵¹ Additionally, there are many other intein-based expression systems that are commercially available that

provide a multitude of options in the implementation of this methodology. This technology should also be of interest to the clinical diagnostics and protein microarray community as it should be possible to directly print antibodies, antibody fragments (e.g., single chain antibodies), as well other as protein scaffolds—such as the FnIII domains⁵² and ankyrin repeat proteins⁵³—that can be recombinantly expressed as intein fusions.

In conclusion, the fabrication of spatially addressable arrays of two different proteins directly from crude cell lysate demonstrated herein suggests that surface-triggered intein ligation is a promising methodology for the development of the next generation of diagnostics and protein arrays, as it will allow recombinant proteins to be directly immobilized or arrayed on a surface from cells that overexpress the protein, thereby eliminating one of the critical bottlenecks—the multiple steps typically required to purify a protein—in the fabrication of bioanalytical devices that use one or more proteins as critical elements of the device.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank the NIH for financial support of this research through grant R01 GM61232 to A.C.

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Figure 1.

Trx conjugation to a hydrazine-terminated SAM by surface-triggered intein ligation of a Trx-intein-ELP fusion, and subsequent anti-Trx mAb binding to immobilized Trx. (A) SDS-PAGE showing bands for purified Trx-intein-ELP (TIE, top band) and intein-ELP (bottom band). (B) SPR curve of immobilization of Trx-intein-ELP to a hydrazine-terminated SAM on gold surface. (C) The dependence of Trx attachment to a hydrazine-terminated SAM, as a function of the solution mole fraction of the COOH-terminated thiol used to install the hydrazine moiety on the surface, as measured by SPR.

Figure 2.

(A) SPR curves showing Trx binding on a 50% cysteine surface using DTT (Trx-DTT) and MPAA (Trx-MPAA) to cleave the intein fusion protein; (B) SPR curve for the immobilization of Trx onto a 50% EG6-COOH surface using EDC/NHS chemistry, followed by antibody binding; (C) SPR data comparing Trx immobilization and antibody recognition for the three methods described in this paper; (D) The anti-Trx mAb to Trx binding ratio, measured by SPR, for the three methods.

Figure 3.

Micropatterning of Trx from buffer (A) and cell lysate (C). (A) Immunofluorescence images of a Trx microarray on a hydrazine-terminated micropattern by surface-triggered intein ligation. The insets in (A) show the corresponding immunofluorescence images for Trx microarrays fabricated by conventional EPL on a micropatterned cysteine-terminated SAM and by EDC/NHS coupling on a COOH-terminated SAM. (B) S/N between pattern and background of the micropatterns fabricated by the three methods from solutions of the proteins in buffer. In (A) and (B), after micropatterning the surface to present the three different reactive groups, the surfaces were then incubated sequentially in target protein (1mg/ml), anti-Trx antibody, and Alexa 647-IgG solutions in HBS-EP buffer. (C) Immunofluorescence images of protein microarrays fabricated on patterned hydrazine surfaces by surface-triggered intein protein ligation from cell lysate. The hydrazine-patterned SAM was incubated in Trx-intein-ELP in *E. coli* lysate that was spiked with 0.1 mg/ml (1 μ M) and 0.001 mg/ml (10 nM, inset) concentrations of the Trx-intein-ELP fusion protein. (D) The S/N between pattern and background as a function of Trx-intein-ELP concentration in buffer and cell lysate. The lower limit of Trx-intein-ELP concentration in cell lysate that provides a $S/N \geq 2$ for micropatterned samples is 0.001 mg/ml (10 nM). The scale bar in all images is 50 μ m.

Figure 4.

Contact spotting of GFP-intein-ELP and Trx-intein-ELP on a hydrazine-functionalized SAM from buffer (A and B) and from cell lysate (C). The different panels in (A) and (B) for both proteins correspond to fields-of-view of columns of microspots of the two different proteins that were printed on a single slide, imaged and subsequently collated to create a composite image. (A) Fluorescence images of printed GFP-intein-ELP. (Panel I) GFP-ELP (1 mg/ml) and (panel II) GFP-intein-ELP (1 mg/ml) printed on hydrazine-functionalized SAM before washing; (panel III) GFP-ELP spot (from panel I) and (panel IV) GFP-intein-ELP spot (from panel II) after washing; (B) Immunofluorescence images of printed Trx-intein-ELP. (Panel I) Trx-ELP (1mg/ml) and (panel II) Trx-intein-ELP (1 mg/ml) after incubation with anti-Trx mAb followed by Alexa 647-IgG (conjugated secondary antibody) and rinsing of the surface with buffer; (C) Fabrication of a protein microarray of GFP-intein-ELP and Trx-intein-ELP fusion proteins. (Panel I) GFP-intein-ELP (5 mg/ml) printed directly from cell lysate and after washing; (Panel II) Trx-intein-ELP (5 mg/ml) printed directly from cell lysate and after washing. The printed Trx was imaged by incubation with an anti-Trx mAb, followed by incubation with Alexa 647- IgG conjugate. The scale bar in all images corresponds to 500 μm .

Scheme 1.

A schematic representation of protein immobilization on a surface directly from cell lysate by hydrazine-mediated surface-triggered intein ligation. The target protein-intein fusion is expressed in *E. coli*, and then reacts with the hydrazine-functionalized surface, which quickly reacts with the C-terminus of target protein directly from cell lysate. The reaction involves the chemoselective attack, by hydrazine, of the thioester bond formed between the C-terminus of the target protein and the N-terminus of the intein domain.

Scheme 2.

A diagram showing the protein ligation process on a surface by three different reaction routes: (A) the reaction mechanism for surface-triggered hydrazine-intein chemistry and conventional cysteine-thioester based expressed protein ligation (EPL); (B). The modification steps performed on each surface for the three ligation methods: EDC/NHS activation (left); surface triggered hydrazine-intein chemistry (middle) and conventional EPL (right).