

General Method for Site-Specific Protein Immobilization by Staudinger Ligation

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Protein microarrays are playing an increasingly important role in the discovery and characterization of protein–ligand interactions. The uniform orientation conferred by site-specific immobilization is a demonstrable advantage in using such microarrays. Here, we report on a general strategy for fabricating gold surfaces displaying a protein in a uniform orientation. An azido group was installed at the C-terminus of a model protein, bovine pancreatic ribonuclease, by using the method of expressed protein ligation and a synthetic bifunctional reagent. This azido protein was immobilized by Staudinger ligation to a phosphinothioester-displaying self-assembled monolayer on a gold surface. Immobilization proceeded rapidly and selectively via the azido group. The immobilized enzyme retained its catalytic activity and was able to bind to its natural ligand, the ribonuclease inhibitor protein. This strategy provides a general means to fabricate microarrays displaying proteins in a uniform orientation.

INTRODUCTION

Protein–protein, protein–DNA, and protein–small-molecule interactions underlie biological chemistry. High-throughput approaches for the discovery and characterization of these various protein–ligand interactions can elucidate complicated biological pathways. In addition, the screening of small-molecule libraries for agonists and antagonists can provide leads for drug development. Recyclable devices that detect biomarkers in body fluids can be used to diagnose human diseases. All of these challenges could be met with the use of protein microarrays (1–7).

Microarrays that present proteins in a uniform orientation exhibit both a markedly greater capacity to bind ligands (8–11) and more reproducible biological function (12) than do protein arrays generated by random immobilization. Microarrays of uniformly oriented DNA have long been produced on a large scale and used with notable success in studies of gene function (13–16). The production of DNA microarrays is made facile by the ease with which oligonucleotides can be prepared by chemical synthesis (17) and incorporated into large fragments by DNA polymerase (18). By comparison, the fabrication of protein microarrays is much more arduous. Notable advances are being made in the chemical synthesis of proteins (19), but this technology is still nascent. The immobilization of natural proteins is complicated by their chemical complexity, as each of the 20 proteinogenic amino acids bears a side chain with distinct physicochemical properties, and the reactivity of the same amino acid can vary according to its position in the three-dimensional structure. Post-translational modifications confer additional diversity (20). Other difficulties result from the intrinsic fragility of proteins, which have modest conformational

stability and can be damaged upon chemical modification. Accordingly, new strategies are needed to immobilize proteins while maintaining their integrity.

Proteins have been immobilized on surfaces, both non-covalently and covalently (21). Noncovalent immobilization has been achieved by physical adsorption (22, 23) and affinity tag-mediated complex formation (24, 25). Covalent immobilization, however, results in more robust arrays. A common strategy, immobilization by attack of the nucleophilic side chain of lysine or cysteine residues, typically proceeds at multiple sites and thus yields an array in which the proteins have a random orientation (26, 27). In contrast, site-specific covalent immobilization, via a unique natural (8, 11, 28) or nonnatural residue, affords a uniformly oriented protein array.

The azido group is chemically inert to the functional groups found in nature. Accordingly, the azido group is being exploited widely by chemical biologists (29), including in the site-specific immobilization of proteins and peptides by the Huisgen 1,3-dipolar azide–alkyne cycloaddition (30–32) and Staudinger ligation (32–34). A limitation, however, has been the difficulty of installing an azido group in a particular position in a protein. Recently, we developed a general method for appending the C-terminus of proteins with an azido group (35). Specifically, we used a bifunctional reagent bearing an α -hydrazino acetamido and azido group to cleave the transient thioester generated on the C-terminus of the target protein fused to an intein, thereby labeling the protein with an azido group on the C-terminus (Scheme 1). This azido group was available for site-specific installation of a fluorophore by the Huisgen 1,3-dipolar azide–alkyne cycloaddition.

Here, we report a general method for the site-specific immobilization of a protein. As a model protein, we choose one of the most thoroughly studied enzymes, bovine pancreatic ribonuclease (RNase A (36); EC 3.1.27.5). The immobilized protein was detected with a standard immunoassay, which does not report on the integrity of the protein. The choice of RNase A provides two rigorous means to assess its structure and function. First, RNase A is an efficient catalyst of RNA cleavage, and its catalytic activity provides a measure of its conformational integrity. Second, the affinity of the ribonuclease inhibitor protein (RI) for RNase A is extremely high (37), and this interaction provides an independent report on the structure

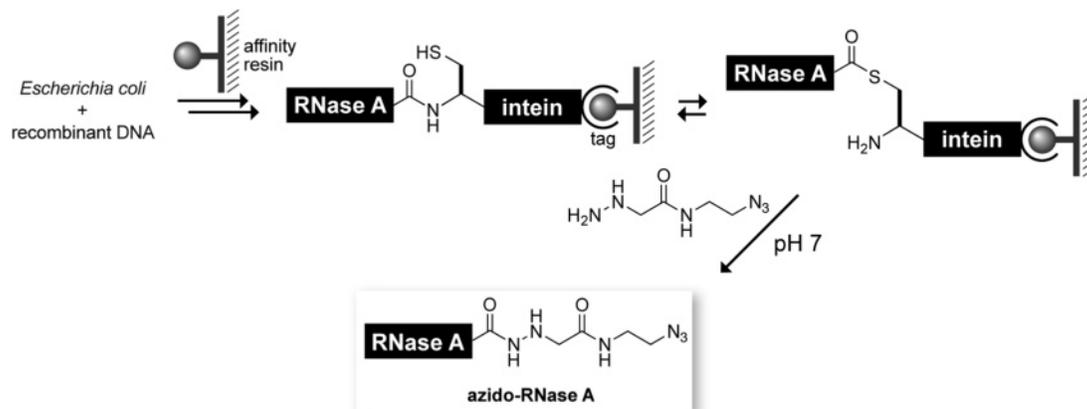
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Scheme 1. Semisynthesis of Azido-RNase A (35)



and function of the immobilized protein. Our strategy was to react an azido-RNase A (Scheme 1) (35) with a phosphinothioester group displayed on self-assembled monolayers (SAMs) of alkane thiols on a gold surface (Scheme 2). The resulting Staudinger ligation (19, 38–40) immobilized the protein in a form that retained its biological function.

MATERIALS AND METHODS

Materials. Silicon chips coated with gold prepared by physical vapor deposition as described previously (41) and were a generous gift from C.-H. Jang. Alkane thiols $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_6\text{OCH}_2\text{CO}_2\text{H}$ and $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_3\text{OH}$ were from Prochimia (Gdansk, Poland). A fluorogenic ribonuclease substrate, 6-carboxyfluorescein-dArU(dA)₂-6-carboxytetramethylrhodamine (6-FAM-dArU(dA)₂-6-TAMRA), was from Integrated DNA Technologies (Coralville, IA). Two-hand AtmosBags were from Sigma-Aldrich (St. Louis, MO).

Azido-RNase A was produced as described previously (Scheme 1) (35). Wild-type RNase A was from Sigma-Aldrich. Human RI was prepared as described previously and was a generous gift from R. J. Johnson and G. A. Ellis. Anti-RNase A rabbit primary antibody was from Biorad International (Kennebunk, ME). AlexaFluor 488-conjugated anti-rabbit secondary antibody was from Molecular Probes (Eugene, OR). Anti-RI chicken primary antibody was from Genetel (Madison, WI). Fluorescein-conjugated anti-chicken secondary antibody was from Abcam (Cambridge, MA).

Instrumentation. Fluorescence measurements for assaying ribonucleolytic activity were made with a QuantaMaster 1 photon-counting spectrofluorimeter equipped with sample stirring (Photon Technology International, South Brunswick, NJ). Immunoassays of immobilized RNase A and RI bound to the immobilized RNase A were visualized with a GeneTac UC4 × 4 fluorescence scanner (Genomic Solutions, Ann Arbor, MI). The optical thickness of SAMs and proteins on the gold chips was determined with a AutoEL ellipsometer (Rudolf Research, Flanders, NJ).

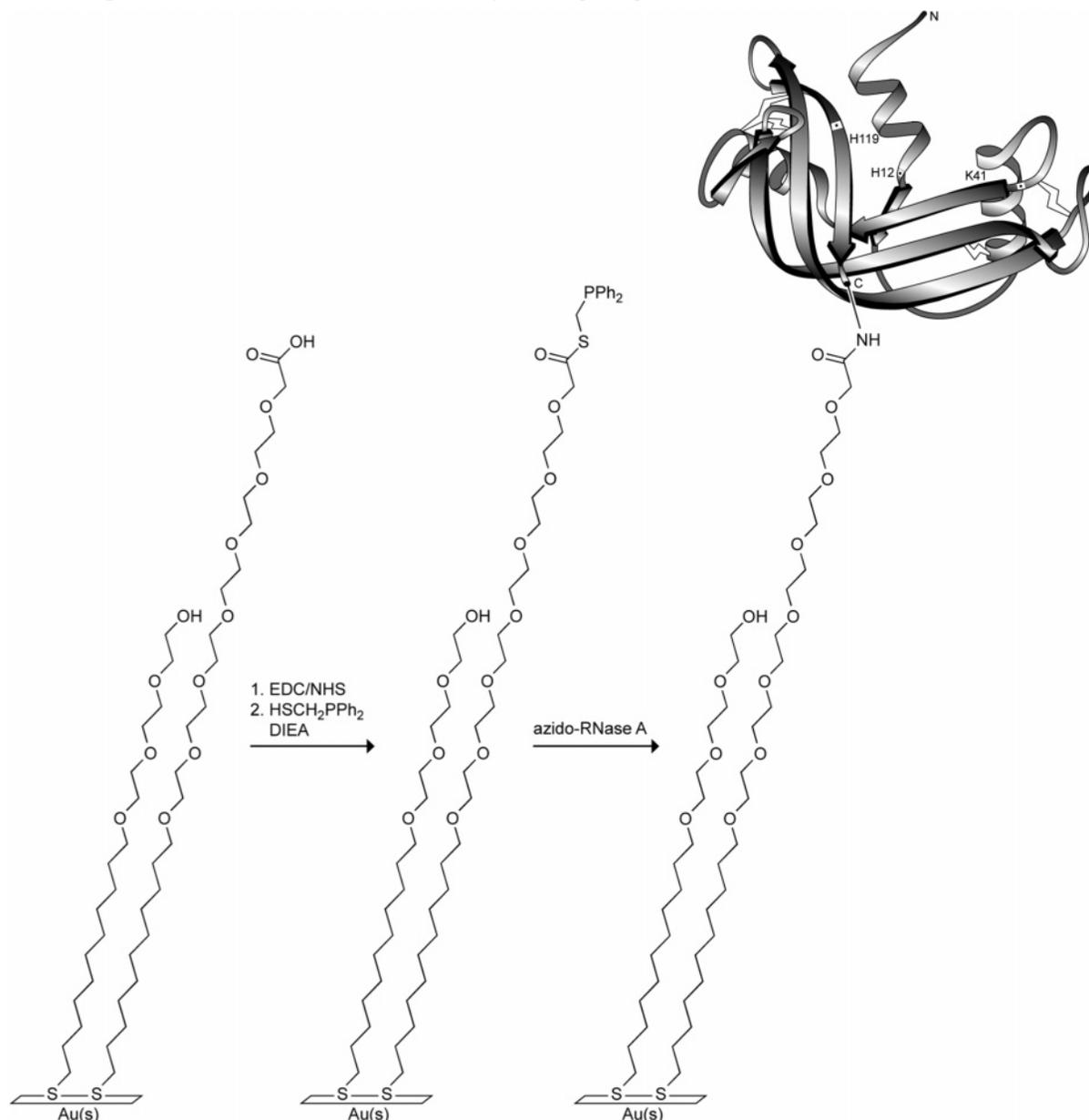
Preparation of Phosphinothioester-Displaying SAMs of Alkane Thiols on Gold Chips. Alkane thiol solutions were prepared by dissolving $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_6\text{OCH}_2\text{CO}_2\text{H}$ and $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_3\text{OH}$ in ethanol to a final concentration of 0.25 mM each. The oligo(ethylene oxide) $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_3\text{OH}$ is included to discourage the nonspecific adsorption of protein (42), which can be problematic (43). Gold chips were cleaned under a stream of Ar(g) and immersed in the alkane thiol solution for at least 18 h. After rinsing thoroughly with ethanol and drying under a stream of Ar(g), the chips were overlaid with an aqueous solution containing *N*-hydroxysuccinimide (NHS, 50 mM) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 200 mM) for 7 min to generate

succinimidyl esters in situ. Diphenylphosphinothioethanol (PPh₂CH₂SH) was synthesized as described earlier previously (44), and was dissolved in anhydrous DMF to a concentration of 0.10 M. *N,N*-Diisopropylethylamine (DIEA) was added to a concentration of 0.12 M. The resulting solution (in a 25 mL flask), succinimidyl ester displaying chips (in a Petri dish containing a water-soaked absorbent paper to serve as a humid chamber), and azido-RNase A (10 μM in 5% v/v DMF(aq)) were placed in a two-hand AtmosBag along with vials containing anhydrous DMF (20 mL/vial), water (20 mL/vial), and 25 mM sodium phosphate buffer at pH 7.5 (20 mL/vial). The AtmosBag was sealed and connected to the house vacuum on one end and an Ar(g) supply on the other end. The air inside the bag was removed by using the house vacuum and flushing twice with Ar(g) before finally filling up the bag with Ar(g). The diphenylphosphinothioethanol-DIEA solution was transferred into a small empty vial, and succinimidyl ester displaying chips were incubated in that vial for 2 h. The phosphinothioester-displaying chips thus produced were rinsed with DMF (2 × 20 mL) and then with water (2 × 20 mL).

Immobilization of Azido-RNase A by Staudinger Ligation. Azido-RNase A (1 μL, 10 μM in 5% v/v DMF(aq)) was incubated on phosphinothioester-displaying chips for 1, 5, 10, and 15 min inside the AtmosBag (Figure 1). The chips were subsequently rinsed with 25 mM sodium phosphate buffer at pH 7.5 (20 mL) and removed from the AtmosBag. They were then incubated in 25 mM sodium phosphate buffer at pH 7.5 (1.0 mL) for 30 min to remove protein bound nonspecifically.

Detection of Immobilized RNase A. A chip displaying immobilized RNase A was overlaid with anti-RNase A rabbit primary antibody (100 μg/mL in 25 mM sodium phosphate buffer at pH 7.5) for 30 min. The chip was then rinsed with 25 mM sodium phosphate buffer at pH 7.5 (2 × 20 mL), and incubated in the same buffer for 15 min. The chip was subsequently overlaid with AlexaFluor 488-conjugated anti-rabbit secondary antibody (2.0 μg/mL in 25 mM sodium phosphate buffer at pH 7.5) for 30 min. Finally, the chip was rinsed with 25 mM sodium phosphate buffer at pH 7.5 (2 × 20 mL), incubated in the same buffer for 15 min, and scanned with a Genomic Solutions GeneTac UC4 × 4 Fluorescence Scanner using a gain of 50.

Ribonucleolytic Activity Assay on Immobilized RNase A. A phosphinothioester-displaying chip was overlaid with azido-RNase A (10 μM in 5% v/v DMF(aq)) in an Ar(g) atmosphere to immobilize RNase A. The chip was rinsed with 25 mM sodium phosphate buffer at pH 7.5 (2 × 20 mL) and then introduced into a vial containing a solution (22 mL) of 0.10 M 2-(*N*-morpholino)ethanesulfonic acid (MES)-NaOH buffer at pH 6.0 containing 0.10 M NaCl. A fluorogenic substrate of RNase A, 6-FAM-dArU(dA)₂-6-TAMRA (10 μL of a 40 μM

Scheme 2. Site-Specific Protein Immobilization on a SAM by Staudinger Ligation^a

^a The three active-site residues and N- and C-termini are indicated explicitly.

solution) was introduced into the vial, and the vial was placed on a shaker. Aliquots (2.0 mL) were withdrawn at known time intervals, and their fluorescence was measured ($\lambda_{\text{ex}} = 493 \text{ nm}$, $\lambda_{\text{em}} = 515 \text{ nm}$). After 1 h, excess wild-type RNase A (20 μL of a 2.8 mg/mL solution) was added to obtain a value of fluorescence after complete cleavage of the substrate. A phosphinothioester-displaying chip was overlaid with wild-type RNase A (10 μM in 5% v/v DMF(aq)), and the assay was performed again. As a negative control, a similar assay was performed on a phosphinothioester-displaying chip which was not overlaid with RNase A. The percentage of the fluorescent substrate cleaved at various time intervals in the negative control was subtracted from the corresponding values for azido- and wild-type RNase A, and plotted against time to generate the graph in Figure 2.

Detection of RI Bound to Immobilized RNase A. Azido-RNase A (1 μL , 10 μM in 5% v/v DMF(aq)) was spotted on phosphinothioester-displaying SAMs on a gold chip for 15 min in an AtmosBag filled with Ar(g). The chip was removed from the bag, rinsed with 25 mM sodium phosphate buffer at pH 7.5

(2 \times 20 mL), and incubated in the same buffer for 30 min to remove nonspecifically bound RNase A. RI (10 μM in 25 mM sodium phosphate buffer at pH 7.5 containing 10 mM DTT) was overlaid on the chip for 10 min at 4 $^{\circ}\text{C}$. The chip was then rinsed with 25 mM sodium phosphate buffer at pH 7.5 containing 10 mM DTT (2 \times 20 mL) and incubated in the same buffer for 15 min.

Immunoassay. After RI binding, the chip was overlaid with bovine serum albumin (2.0 mg/mL in 25 mM sodium phosphate buffer at pH 7.5) for 15 min. The chip was rinsed with 25 mM sodium phosphate buffer at pH 7.5 (2 \times 20 mL), and subsequently incubated with anti-RI chicken primary antibody (12.5 $\mu\text{g}/\text{mL}$ in 25 mM sodium phosphate buffer at pH 7.5) for 15 min. The chip was rinsed with 25 mM sodium phosphate buffer at pH 7.5 (2 \times 20 mL), incubated in the same buffer for 15 min, and subsequently overlaid with fluorescein-conjugated anti-chicken secondary antibody (1.5 $\mu\text{g}/\text{mL}$ in 25 mM sodium phosphate buffer at pH 7.5) for 15 min. The chip was rinsed with 25 mM sodium phosphate buffer at pH 7.5 (2 \times 20 mL), incubated in the same buffer for 15 min, and then scanned with

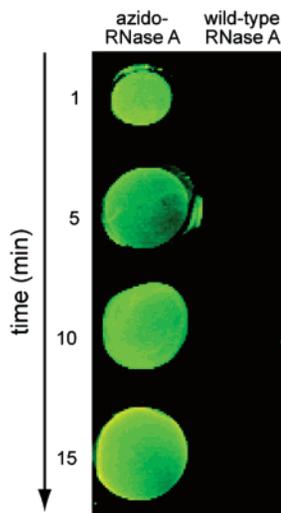


Figure 1. Time course and specificity of immobilization of azido-RNase A. Azido-RNase A and wild-type RNase A (each 10 μM) were spotted on a phosphinothioester-displaying chip for various times. The chip was overlaid with an anti-RNase A primary antibody (10 $\mu\text{g}/\text{mL}$) and a secondary antibody conjugated to AlexaFluor 488 (2 $\mu\text{g}/\text{mL}$).

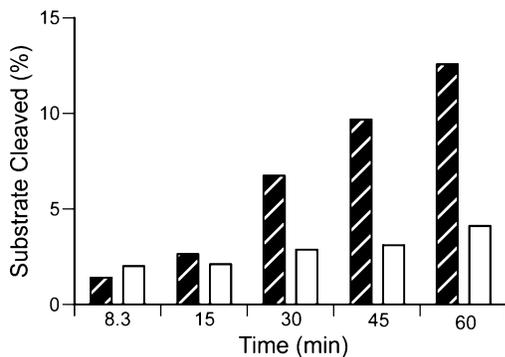


Figure 2. Catalytic activity of immobilized RNase A. Phosphinothioester-displaying chips were incubated with azido-RNase A (hatched bars) or wild-type RNase A (open bars), and assayed for ribonucleolytic activity by using a fluorogenic substrate.

a Genomic Solutions GeneTac UC4 \times 4 fluorescence scanner using a gain of 50.

Ellipsometry. Ellipsometric measurements were performed to determine the optical thicknesses of the SAMs, RNase A, and RI on the gold surface. The measurements were performed with a wavelength of 632 nm and an angle of incidence of 70°. The optical thickness reported is the average from seven different surface locations. A slab model was used to interpret these constants in which the slab (SAM and protein) was assumed to have an index of refraction of 1.46.

RESULTS AND DISCUSSION

Generation of Phosphinothioester-Displaying SAMs on Gold. SAMs of alkane thiols on gold were prepared by incubating gold-coated silicon chips in an ethanolic solution containing equimolar quantities of $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_6\text{OCH}_2\text{CO}_2\text{H}$ and $\text{HS}(\text{CH}_2)_{11}(\text{OCH}_2\text{CH}_2)_3\text{OH}$. Subsequently, the chips were overlaid with an aqueous solution of EDC/NHS to produce succinimidyl ester-displaying chips. Diphenylphosphinomethanethiol was synthesized in multigram quantities by a procedure described previously (44), and was reacted with succinimidyl ester-displaying chips to generate phosphinothioester-displaying chips. The resultant phosphinothioester-displaying SAMs of alkane thiols on gold surfaces provide a highly

homogeneous surface for performing the Staudinger ligation. These surfaces are significantly better defined than that of a glass slide, which had been used previously for Staudinger ligation (33, 34, 45), thereby enabling the use of high-precision techniques such as ellipsometry, surface plasmon resonance (46), and liquid crystal methods (11, 47) for the detection of ligand binding.

Azido-RNase A Immobilization by Staudinger Ligation. Azido-RNase A (in 5% v/v DMF(aq)) was incubated on phosphinothioester-displaying chips for immobilization by Staudinger ligation (Scheme 2). A time course of immobilization of azido-RNase A is depicted in Figure 1. The immobilized RNase A was detected by an immunoassay utilizing a primary anti-RNase A antibody and a fluorescent secondary antibody. Protein immobilization proceeded rapidly, with a significant fluorescence signal being clearly observable after only 1 min. The rapidity of this reaction is reminiscent of that for the immobilization of azido-peptides to glass slides with a phosphinothioester-mediated Staudinger ligation (33).

Exposure of a protein to a phosphinothioester-displaying chip could lead to undesirable protein immobilization via a side chain of the protein. For example, RNase A has 10 lysine residues, any one of which could attack the phosphinothioester via its nucleophilic ϵ -amino group, thereby resulting in random immobilization. Additionally, the protein could be immobilized noncovalently onto the surface by hydrophobic interactions with the phenyl groups of the phosphinothioester. To probe for these possible immobilization routes, wild-type RNase A was incubated adjacent to azido-RNase A on phosphinothioester-displaying SAMs on gold. No significant immobilization of wild-type RNase A was observed during the time scale required for substantial azido-RNase A immobilization (Figure 1), thereby establishing that the immobilization of azido-RNase A proceeded exclusively via the azido group. Longer incubation times (>1 h) did result in detectable immobilization of wild-type RNase A (data not shown), highlighting the benefit of the rapidity of the Staudinger ligation.

Attempts to immobilize azido-RNase A in purely aqueous solutions of various pH were unsuccessful (data not shown). The presence of a small quantity of polar organic solvent (here, 5% v/v DMF) is required, perhaps for adequate solubilization of the immobilized phosphinothioester. A version of the Staudinger ligation has been used to immobilize proteins in purely aqueous solutions (34), though this method requires a long incubation period (4 h) and installs a racemic nonnatural moiety—a phosphine oxide—into the immobilized protein.

Activity of Immobilized RNase A. Enzymatic activity assays were performed with phosphinothioester-displaying chips incubated with azido-RNase A for 10 min. These chips were incubated in solutions containing 6-FAM-dArU(dA)₂-6-TAMRA, which is a fluorogenic substrate for RNase A (48). After 1 h, the fluorescence intensity from a chip incubated with azido-RNase A had increased substantially relative to one incubated with wild-type RNase A (Figure 2). Thus, the azido-RNase A was immobilized preferentially, and immobilization did not destroy its enzymatic activity.

Binding to Immobilized RNase A. A phosphinothioester-displaying chip was overlaid with azido-RNase A, and subsequently overlaid with RI. Another phosphinothioester-displaying chip was incubated with wild-type RNase A, and then overlaid with RI. Both chips were assayed for the presence of RI by an immunoassay that involved a primary antibody to RI and a fluorescent secondary antibody. The chip that was overlaid with azido-RNase A had a high fluorescence signal, whereas the chip incubated with wild-type RNase A produced negligible fluorescence (Figure 3A). This result demonstrated that the RI detected on the surface was bound to

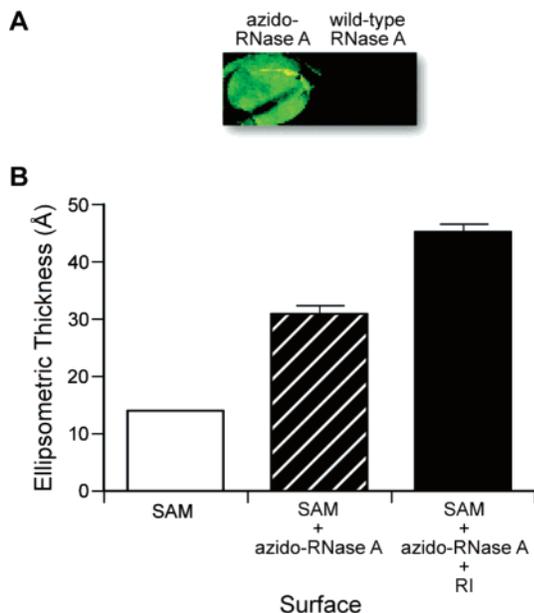


Figure 3. Binding of RI to immobilized RNase A. (A) Immunoassay with a primary anti-RI antibody and a secondary antibody conjugated to fluorescein. (B) Ellipsometric thickness before and after incubation with azido-RNase A, and after the addition of RI.

the immobilized RNase A, and was not binding to the surface nonspecifically.

Ellipsometric measurements corroborated these findings (Figure 3B). Upon incubation of azido-RNase A on phosphinothioester chips for 10 min, the ellipsometric thickness of the surface increased by 16.9 Å. Exposure to RI resulted in a further increase of 14.3 Å. That the increase in thickness upon RI binding was less than the increase in thickness upon azido-RNase A immobilization is surprising, as RI (molecular mass ~51 kDa) is a larger protein than RNase A (molecular mass ~14 kDa). One explanation is that the immobilization of RNase A via its C-terminus forces RI to bind obliquely to the surface, causing a smaller increase in the thickness than expected from lineal binding. Another explanation is that the binding of RI molecules to a few immobilized RNase A molecules creates steric hindrance to other incoming RI molecules. Consequently, not all of the immobilized RNase A molecules would form a complex with RI, producing a smaller than expected increase in ellipsometric thickness. To obtain additional information, we measured the ribonucleolytic activity of the immobilized RNase A after incubation with excess RI. Only half of the activity was lost, consistent with steric hindrance preventing half of the RNase A molecules from forming a complex with RI.

SUMMARY

We have developed a general strategy for the site-specific immobilization of a protein on SAMs on a gold surface. Immunoassays, assays of enzymatic activity, and ellipsometric measurements revealed that the immobilized protein retained its structure and function. We envisage numerous applications of our strategy, which could be applicable to any protein. For example, we expect that our strategy could be useful for the high-throughput screening of ligands, in diagnostic applications that detect disease markers in clinical samples, and in industrial processes that utilize immobilized enzymes as recyclable catalysts (49).

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