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Communication

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Self-Catalyzed Immobilization of GST-Fusion Proteins for Genome-Encoded Biochips

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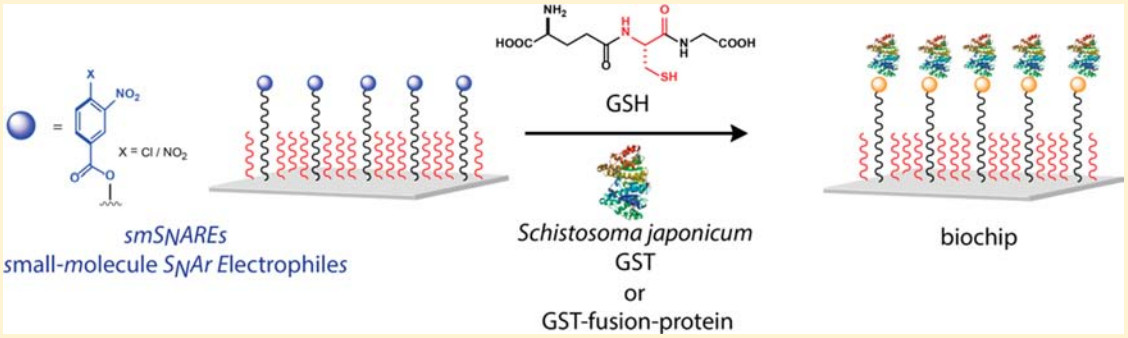
Supporting Information



ABSTRACT: With the surge of proteomic information that has become available in recent years from genome sequencing projects, selective and robust technologies for making protein biochips have become increasingly desirable. Herein, we describe the development of small-molecule SNAr electrophiles (smSNAREs), a new class of capture probes that enables a selective, single-step immobilization for protein biochips. This enzymology-driven approach rides on the binding and catalytic mechanism of *Sj*GST. We have designed and synthesized mechanism-based substrate analogs **3**, **4**, and **5** as electrophilic precursors for conjugation of glutathione S-transferase (GST) or any of its fusion proteins. Upon evaluating the conjugation of these probes to glutathione in the presence of *Sj*GST via UV−visible spectroscopy (UV−vis) and LC-MS techniques, we found that **3**, **4**, and **5** were transferable to GSH. Through the anchoring of alkyne **5** as a smSNARE probe on glass surface, we demonstrate the single-step, self-catalyzed immobilization of *Sj*GST. Fluorescence imaging quantitatively revealed an 18-fold increase in selective binding of *Sj*GST over random orientations (due to nonspecific binding) of the protein. Binding between GST and smSNARE surface is robust and does not reverse upon adding up to 100 mM GSH. Further, a 6-fold increase in resolution for the smSNARE surface probe was observed over commonly employed commercially available GSH-epoxy surfaces. Detailed control experiments revealed insights into the reversibility of binding and catalysis of GSH to form conjugation products with **5** in the presence of the enzyme. As an application of this protein capture technology, we printed alkaloid biosynthesis enzyme, isonitrile synthase (IsnA), to result in a biochip. Because proteins bearing a GST-fusion purification tag are commonly created through the pGEX expression system, these findings show broad potential applicability to genome-wide studies and proteomic platforms.

Miniaturized biochips have revolutionized system-wide, high-throughput studies of biological events.[1−3] Rapid increase of available genomic information during the past two decades has inspired the development of robust biochip technologies for the elucidation of biochemical functions of unknown proteins in a miniaturized format.[4−6] Among these methods, label-free conjugation techniques, especially of commonly employed fusion proteins, add tremendous power to the toolbox of chemical biologists.[7] Label-free conjugation through controlled fundamental mechanisms of protein-side chain modifications is extremely powerful, yet challenging.[8−13] Enzyme-catalyzed approaches offer unique advantages over nonenzymatic chemical modifications due to the fact that selectivity of conjugation is exquisitely programmed thereby minimizing randomness and nonspecific modifications.[14−16] Such techniques eventually result in superior readability of data sets irrespective of nature of the application. We herein report the development of a new enzyme-catalyzed approach to selectively conjugate proteins.

Through this strategy, glutathione S-transferase (GST) or its fusion proteins are printed in a single step, utilizing the dual properties of catalysis and binding of GST (Figure 1). The S_NAr (substitution-nucleophilic aromatic) reaction of Glutathione S-Transferase (GST) offers a bioconjugation mechanism to selectively print proteins as biochips with controlled orientation. We describe herein the development of mechanism-based *small-molecule-SNAREs* (S_NAr electrophiles), a new class of capture probes that lay the foundation for an enzymatic strategy, for protein bioconjugation (and different from SNAP

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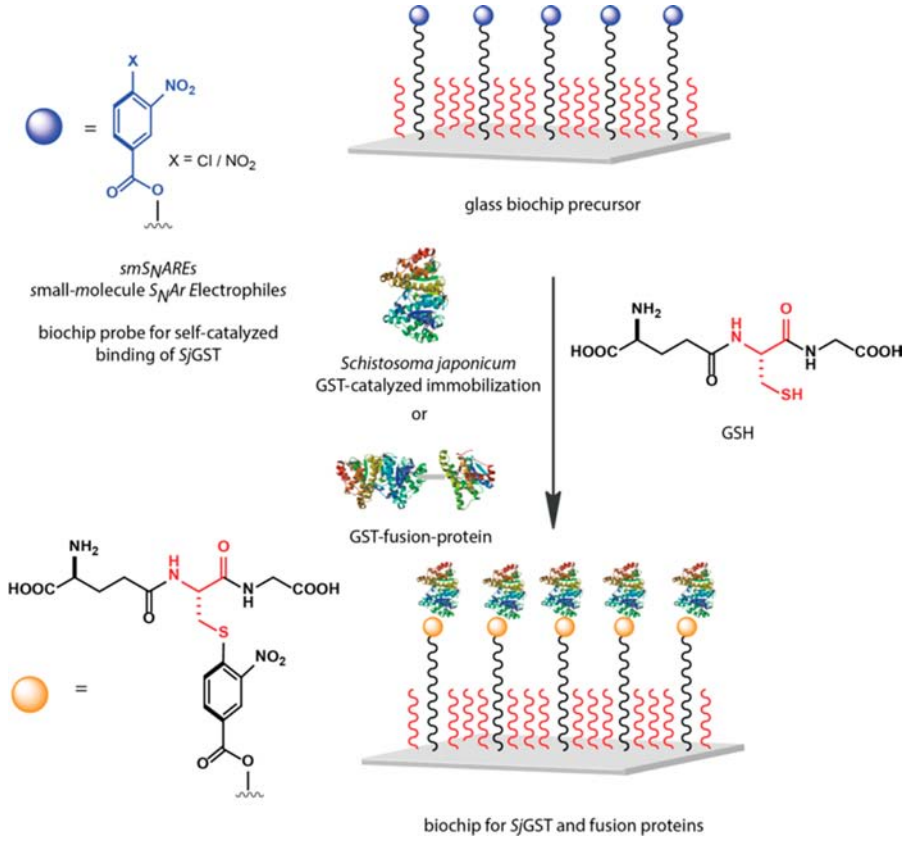
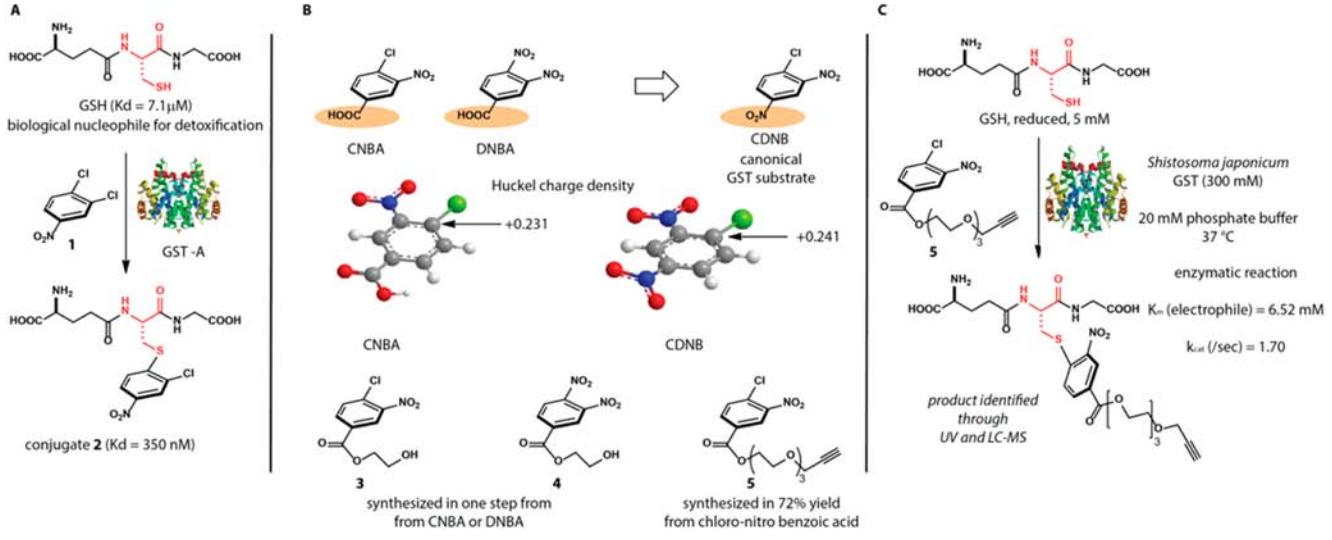


Figure 1. Outline of smSNARE-captured bioconjugation strategy. The self-catalysis and binding to immobilize GST in their native orientation is represented through participation of GSH as a nucleophile, smSNARE electrophile, and GST or its fusion protein as the enzyme.

Scheme 1[a]



[a](A) GSH-conjugation to **1**, the first step in mercapturic acid biosynthesis catalyzed by GST-A. (B) Design and electronic tuning of aromatic substituents for the synthesis and evaluation of smSNARE probes **3**, **4**, and **5**. Hückel charge densities are calculated using Chem 3D. (C) *In vitro* enzymatic incorporation of smSNARE probe **5** to GSH.

Receptors[17]). By combining the organic chemistry of this smSNARE-capture strategy with molecular biology of pGEX expression vectors, alkaloid biosynthesis enzyme IsnA is selectively printed as a biochip in one step.

GSTs (E. C. 2.5.1.18) are a multigene family of enzymes that are widely used as fusion tags to selectively isolate proteins from proteomic mixtures.[18] These enzymes are unique, because in addition to binding to glutathione (GSH), a useful feature for protein purification, they act as catalysts for the conjugation of GSH to a wide variety of electrophiles.[19−23] Nature has evolved a suite of GSTs for detoxification chemistries that comprise the mercapturic acid biosynthesis pathway in eukaryotes. Binding studies by Mannervik (Scheme 1A) using equilibrium dialysis methods on GST-A class enzymes with

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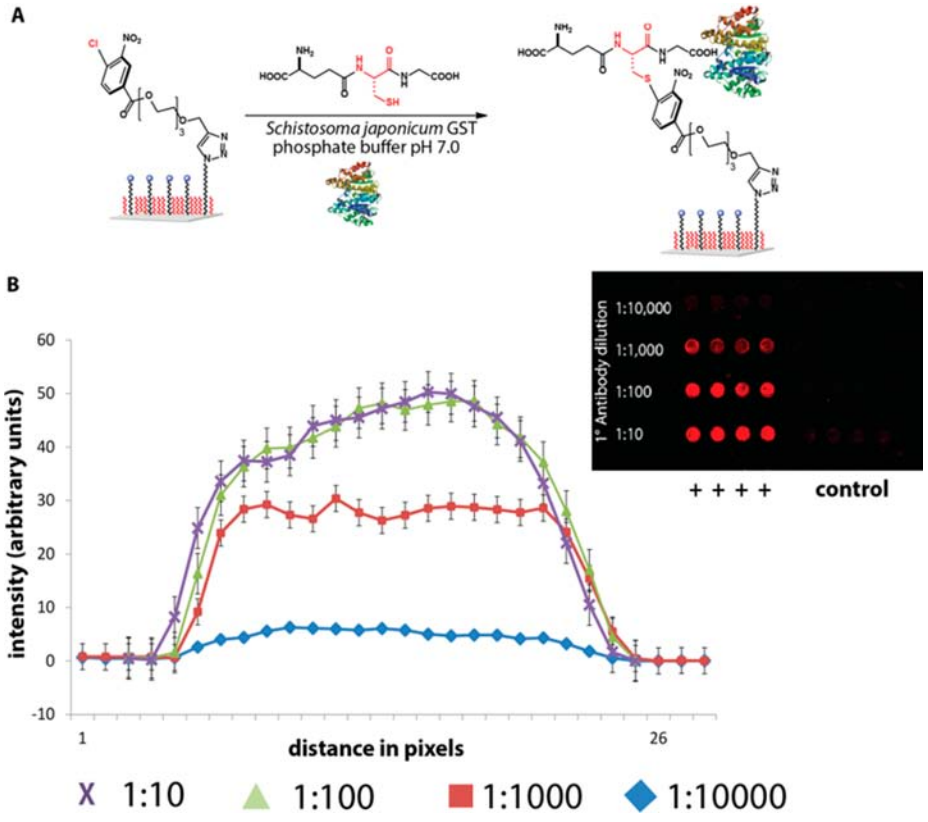
Scheme 2[a]



[a](A) smSNARE surface probe capture of *Sj*GST. (B) Plot consisting of signal intensities for fluorescence image of immobilized *Sj*GST biochip (ImageJ was used for quantification). Inset: biochip image of *Sj*GST. + = GSH, GST are spotted followed by primary and secondary antibody. Control = spots where all but primary antibody were added.

GSH and a 3,4-dichloro-1-nitrobenzene electrophile (**1**) reveal that GST-A binds the GSH-conjugate **2** with >20-fold higher affinity than GSH alone (K_d = 350 nM versus K_d = 7.1 μM, respectively).[20] Based on this observation, we hypothesized that the development of a "first-generation" library of transferable analogues (derived from the 1-chloro, 2,4-dinitrobenzene (CDNB) scaffold) may lead to a new self-catalytic class of GST-binding surface probes. Furthermore, because fusions of *Schistosoma japonicum* GST (*Sj*GST) contain catalytic domains similar to GST-A class enzymes, we projected that our mechanism-based probes may trigger a self-catalyzing capture event for uniformly and selectively anchoring GST-fusion proteins to create biochips.

We designed, synthesized, and evaluated *in vitro* a first-generation class of smSNARE probes based on the canonical CDNB scaffold (Scheme 1B). To permit tethering with appropriate linkers, the C4 nitro group of CDNB was exchanged for an ester functionality, based on modification of the carboxylic acid group of 4-chloro-3-nitrobenzoic acid (CNBA) or 3,4-dinitrobenzoic acid (DNBA). We aimed to maintain the electrophilicity of C1 in order to facilitate the nucleophilic aromatic substitution (S_NAr) between GSH and the electrophilic probes. Hückel charge densities at the chloro position for CDNB (+0.241) and CNBA (+0.231) were comparable, indicating that the electrophilic nature at C1 was preserved upon replacement of the C4 nitro group with a carbonyl functionality. The synthesis and characterization of three GST substrate analogues (compounds **3**, **4**, and **5**) followed straightforward methods (see SI). The *Sj*GST-catalyzed conjugation reactions of these analogues to GSH were evaluated. All three substrates displayed favorable solubility for bioconjugation and the presence of an alkyne in smSNARE **5** enabled facile surface monolayer construction through click chemistry.

Upon subjecting alkyne **5** (0.5 mM) to conjugation with GSH (5 mM) in the presence of 300 nM *Sj*GST in 20 mM phosphate buffer, pH 7.0 at 25 °C, we confirmed the formation of **GSH-5** (Scheme 1C). The UV−visible spectroscopic profile of the conjugation reaction between GSH and compound **5** showed a new absorption peak appearing at ∼360 nm, analogous to the absorption peak at 340 nm known to appear when the canonical substrate CDNB is conjugated to GSH.[24] The extinction coefficient at 356 nm (ε_{356}) is 983 $M^{-1}\cdot cm^{-1}$ for conjugate **GSH-5** (see Table S1). The presence of **GSH-5** was confirmed through detection of an LC-MS peak at m/z = 643, consistent with the structure shown in Scheme 1C (Figure S2). Similarly GSH conjugates of **3** and **4** were detected through LC-MS analysis (Figure S5 and S8). Preliminary profiles of reaction kinetics were obtained through a Lineweaver−Burk plot analysis for conjugation reactions involving **3**, **4**, and **5**, respectively (Figures S3, S6, and S9). Transferability of these analogues correlated well with reports on other substrate analogs of CDNB.[25,26] While the alcohol-bearing **3** displayed superior transferability to GSH under *Sj*GST catalysis, we chose to study surface monolayer formation with alkyne **5** due to the availability of an extended "clickable" tether. Azide-coated glass surfaces were prepared by sequential treatment of ordinary glass microscope slides with aminopropyl triethoxy silane

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(APTES) and 6-azido-hexanoic acid. To confirm the presence of azide groups on the slide, alkyne-bearing fluorescent nanoparticles were conjugated to the azide-modified surface under standard Cu(I)-catalyzed triazole formation conditions (Figure S10). A greater signal enhancement was obtained for nanoparticles clicked to smSNARE surfaces than using fluorescent small-molecule click partners. Contact-angle microscopy (CAM) showed the increase in surface hydrophobicity through this functionalization (contact angle = 58.4°). Alkyne-containing smSNARE probe **5** was clicked to the azido surface under similar conditions.

Conditions for the smSNARE-captured protein immobilization were extended from the *in vitro* enzymatic reaction previously performed in solution (Scheme 1C). The results of this study are illustrated in Scheme 2. Enzymatic reaction solution containing GSH (5 mM) and *Sj*GST (300 nM) in phosphate buffer (20 mM, pH 7.0) was applied to the smSNARE probe surface (Scheme 2A). Similar to techniques used in standard Western blots, the extent of the self-catalyzed conjugation and binding between the smSNARE probe, GSH, and *Sj*GST was visualized by fluorescence immunolabeling with a mouse anti-GST 1° and a fluorescently labeled 2° detection antibody. Reproducibility in protein immobilization was evident between each row as the spots are quadruplicate immobilizations, averaged to give intensity values that are plotted in Scheme 2B. While the dilution of 1° antibody was varied by factors of 10 from 1:10 to 1:10 000, the fluorescently labeled 2° antibody was used at a constant dilution (1:200). Fluorescent spots were visualized with a Typhoon gel imager (532 nm excitation) and quantified with ImageJ software, showing a consistent increase in intensity (Scheme 2B) with increasing concentration of mouse anti-GST 1° antibody. The images shown were quantified and plotted as displayed in Scheme 2B. Controls without added *Sj*GST (shown in Scheme 2B, right) displayed no residual fluorescence signal, consistent with our expectation that catalysis and binding requires the protein. Scalability was not tested in this pilot study, though we envision programming of this immobilization to be a smooth operation at larger amounts of proteins.

In order to quantitate the level of GST immobilization achieved through this smSNARE-capture step and to compare it to the level of nonspecific adsorption of GST to glass surfaces via different covalent and noncovalent binding methods, a series of control experiments was carried out. Measured intensities of immobilized *Sj*GST were obtained through methods identical to the previous biochip analyses (Scheme 2B). Lanes 1−4 (Figure 2) show immobilization where the presence or absence of *Sj*GST and GSH was tested individually. The signal intensity in lane 2 confirmed that *Sj*GST is required for fluorescence. Lane 3 shows the *Sj*GST nonspecific binding to smSNARE probes without GSH conjugation, with the intensity ratio of lane1:lane 3 showing the extent of selective smSNARE-capture. An 18-fold increase in immobilization efficiency is directly attributable to smSNARE-captured self-catalysis. We attribute this 18-fold increase in *Sj*GST binding to the greater binding recognition (lowering of K_d) of *Sj*GST to the product of the conjugation reaction (corroborating with reversibility study, see below). The lack of fluorescence intensity in lane 4 confirmed that the antibodies were not causing false positive fluorescence signals. Results from lanes 1−4 indicated that smSNARE-GSH conjugate, the product of the GST-catalyzed reaction, has a much lower K_d than free GSH. We wondered whether competitive binding might be an

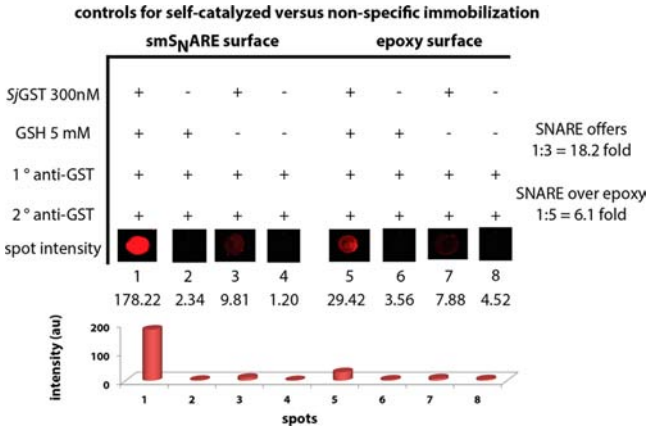


Figure 2. Plot consisting of signal intensities for fluorescence image of immobilized *Sj*GST (ImageJ was used for quantification). Control experiments comparing smSNARE probe surface to epoxy surfaces.

issue with the 5 mM concentration of GSH present during the immobilization step. Also, as a corollary, we wondered if there is a range of GSH concentration that might render reversibility to the biochip formation step. In order to address this, we carried out, post-immobilization, incubation of GST-labeled smSNARE surfaces with either 100 mM GSH in phosphate buffer (20 mM, pH 7.0) or 1% sodium dodecyl sulfate in glycine buffer (200 mM, pH 2.0). In neither case did we observe reversal of the binding of GST to the surface, indicated by persistence of fluorescent signals at the same intensity before and after these incubations.[27] These observations point to the fact that binding efficiency between GSH−smSNARE conjugate and *Sj*GST increases drastically upon completion of a single catalytic turnover, leading to a near-complete binding of product. Surface plasmon resonance (SPR) studies in the future may address quantitation of these observations. With respect to reversibility, smSNARE technology offers a complementary option to elegant mechanisms of reversible GST immobilization reported by the Maynard group.[28]

Next, we addressed fundamental comparisons between smSNARE-based immobilization and other commonly employed *Sj*GST binding methods, such as GSH-modified epoxy surfaces. For GST over epoxy-GSH surface, the immobilization steps causing printing of proteins were performed consistently with those recommended by the manufacturer (see SI). Also, concentrations and duration for immobilization on epoxy surface were adjusted to account for greater surface coverage of epoxides than smSNARE slides. *Sj*GST does indeed bind to GSH-coated epoxy slides, as shown by the image in lane 5 of Figure 2. Upon comparing lane 5 to lane 1, it was found that smSNARE-capture strategy leads to a 6-fold improvement over the standard GSH-epoxy surface for immobilization of GST. Also, when no GSH is present (lane 7), *Sj*GST binds to epoxy slides in random orientations through nonselective modification of its surface nucleophilic residues (e.g., lysine or cysteine), indicated by low fluorescence intensity. The intensity ratio of lane 1:lane 3 (178.22:9.81 = 18.1) versus that of lane 5:lane 7 (29.42:7.88 = 3.7) reflects the magnitude of signal-to-noise for smSNARE versus epoxy surfaces, respectively. The smSNARE probes therefore offer improved efficiency for selective immobilization over regular epoxy surfaces. These observations are consistent with previous studies, such as Mahal et al.'s report that lectins oriented to succinimidyl-GSH surfaces display a 17-fold increase in efficiency of protein immobiliza-

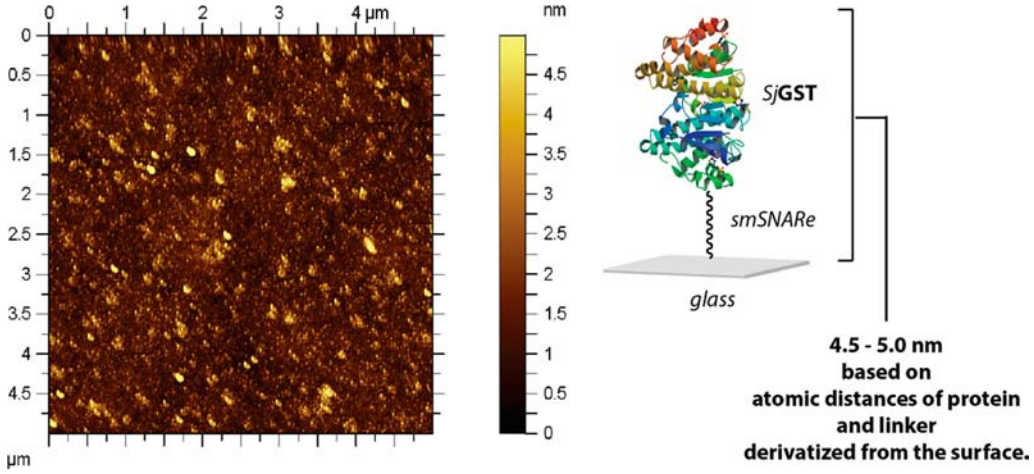


Figure 3. AFM image showing morphology (left) and estimation of surface dimensions for immobilized *Sj*GST (right).

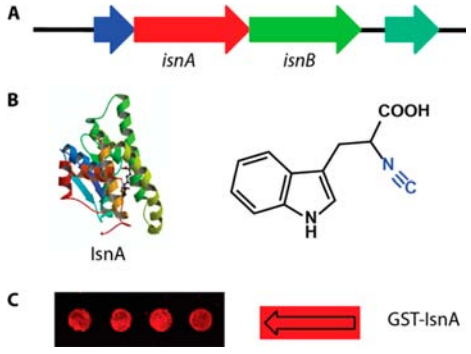
tion.[29] In this context, these controls correlate with findings reported by Maynard et al. on GST immobilization through chemically modified GSH analogues.[28]

Atomic force microscopy (AFM) was employed to provide insight into the surface morphology of GST-immobilized glass surfaces prepared via the S_NAr-E-capture method. Scheme 2E shows an AFM image of a *Sj*GST-immobilized slide prepared with the same concentrations of GSH and GST as described above. A 5 × 5 μm^2 scan area was chosen in order to show consistency of the immobilized biomolecules. This image shows that the surface height varies between 0 and 5 nm depending on the exact location on the slide. Using X-ray structure data and a protein biophysical estimation suite (Phyre 2.0), the diameter of surface-immobilized *Sj*GST (see SI) was calculated to be between 4.5 and 5 nm, which corresponds to the morphology observed in the AFM image.

As an application of this smSNARE-capture technology, we chose to immobilize an eDNA-derived (DNA recovered from an environmental source) alkaloid biosynthesis protein of interest (Scheme 3). The cDNA for *isnA* (responsible for biosynthesis of L-tryptophan isonitrile[30]) was cloned into a pGEX-3X vector and expressed under IPTG-induction to produce GST-IsnA (see SI). The smSNARE-capture strategy was employed for the immobilization of GST-IsnA under identical conditions as described for *Sj*GST. Scheme 3C shows a fluorescence image of an immobilized GST-IsnA array that has been treated with a mouse anti-GST 1° and a fluorescently labeled 2° detection antibody. Control experiments were run such that GST-tag bearing proteins are detected specifically by the immunolabeling technique. Therefore, any of the proteins from pGEX-3X vector (without the IsnA insert) containing a fully expressed GST-tag will be detected. However, the SDS-PAGE gel (SI) confirmed that the overexpressed IsnA-GST fusion was the dominant GST-containing protein for "pull-down" experiments after purification. The IsnA-GST fusion was captured by both smSNARE surface and GSH-epoxy slides; however, it was noted that the GSH-epoxy slides showed greater background binding reflected by the intensity shown by the fluorescent antibodies.[27] This may imply that a significant portion of the observed fluorescence of isnA-GSH-epoxy system is attributable to nonspecific binding of the antibodies, whereas IsnA-smSNARE surfaces suffer from this problem less, and a more efficient IsnA capture occurs for the latter system. The successful creation of this biochip opens the door for using smSNARE-capture technology for building biocatalytic chips with other genomic proteins and for lab-on-a-chip applications. Future experiments will focus on deciphering enzyme action on biochips of IsnA in addition to other genome-wide protein profiling applications.

In conclusion, we report a new class of smSNAREs for a single-step immobilization of GST-fusion proteins. Our initial findings show that the transferable analogue **5** can serve to irreversibly capture proteins with a 6-fold higher selectivity over epoxy surfaces. The smSNARE probes used for this step offer a mechanism-based protein immobilization tool toward proteomic applications. Generally, in comparison to phenomenally useful DNA biochips,[31] protein biochip construction presents unique challenges due to selectivity and preservation of enzyme function. The SNArE-capture strategy is selective because it takes advantage of the native catalysis of GSTs. Further the fact that catalysis and binding occur in one step obviates the need to prefunctionalize the surface with GSH. The latter feature is a useful property for biochip applications, since GSH can participate in aerobic chemistry (oxidation and elimination) that causes degradation of the surface. In addition these surfaces offer superior resolution over existing commercial epoxy platforms. This strategy is envisioned as broadly applicable to slides, surfaces, nanoparticles, and other

Scheme 3[a]



[a](A) Genome-encoded metabolic enzymes in alkaloid biosynthesis. pGEX-3X-*isnA* was constructed for protein immobilization. (B) Function of e-DNA-derived IsnA:biosynthesis of L-tryptophan isonitrile. (C) SNARE-captured simultaneous self-catalysis and binding to immobilize GST-IsnA.

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biomedical sensors that potentially can be layered with smSNAREs. Proteomics applications also call for generality for construction of ELISA-like "pull down" assays for a wide range of proteins. The tool developed here anchors any gene of interest to be bioconjugated in a single step after translation to corresponding GST-fusion proteins. Therefore, future studies will probe application of this strategy in ELISA-like binding assays of proteomic mixtures.

■ ASSOCIATED CONTENT

Supporting Information

Methods for the synthesis and spectral data of smSNARE probes **3**, **4**, and **5**. Data for enzymatic evaluation and detection of GSH conjugates of **3**, **4**, and **5**. Microscopy images of surfaces, general protocols for immobilization, and additional controls for GST immobilization. Cloning and biochemical steps for preparation and expression of pGEX-isnA. 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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