

# Immobilization of oriented protein molecules on poly(ethylene glycol)-coated Si(111)

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A high-density poly(ethylene glycol) (PEG)-coated Si(111) surface is used for the immobilization of polyhistidine-tagged protein molecules. This process features a number of properties that are highly desirable for protein microarray technology: (i) minimal nonspecific protein adsorption; (ii) highly uniform surface functionality; (iii) controlled protein orientation; and (iv) highly specific immobilization reaction without the need of protein purification. The high-density PEG-coated silicon surface is obtained from the reaction of a multi-arm PEG (mPEG) molecule with a chlorine terminated Si(111) surface to give a mPEG film with thickness of 5.2 nm. Four out of the eight arms on each immobilized mPEG molecule are accessible for linking to the chelating iminodiacetic acid (IDA) groups for the binding of Cu<sup>2+</sup> ions. The resulting Cu<sup>2+</sup>-IDA-mPEG-Si(111) surface is shown to specifically bind 6x histidine-tagged protein molecules, including green fluorescent protein (GFP) and sulfotransferase (ST), but otherwise retains its inertness towards nonspecific protein adsorption. We demonstrate a particular advantage of this strategy: the possibility of protein immobilization without the need of prepurification. Surface concentrations of relevant chemical species are quantitatively characterized at each reaction step by X-ray photoelectron spectroscopy (XPS). This kind of quantitative analysis is essential in tuning surface concentration and chemical environment for optimal sensitivity in probe-target interaction.

**Keywords:** Cu<sup>2+</sup>-IDA / High density PEG immobilization / Inert surface / Protein microarray

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## 1 Introduction

Since the successful application of DNA microarray technology in biomedical research, [1–5], much attention has been devoted to the development of similar technology for proteins [6–11]. Direct analysis of protein expression is essential because mRNA expression level is poorly correlated with actual proteins due to post-translational

modification. Besides, the function and activity of the majority of protein molecules remain unknown at the present time. Compared to oligonucleotides or cDNAs, protein microarray technology requires much more sophisticated surface engineering to maintain optimal conformation of immobilized protein molecules and to minimize nonspecific adsorption.

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**Abbreviations:** AFM, atomic force microscopy; ATR, attenuated total reflectance; BE, binding energy; DEIDA, diethyl iminodiacetate; DSC, disuccinimidyl carbonate; GFP, green fluorescent protein; IDA, iminodiacetic acid; IPTG, isopropyl-beta-D-thiogalactopyranoside; MIR-FTIR, multiple internal reflection-Fourier transform infrared; mPEG, multi-arm PEG; NTA, nitrilotriacetic acid; ST, sulfotransferase; XPS, X-ray photoelectron spectroscopy

Ideally, we would like the surface chemistry for protein microarrays to meet the following criteria: (i) the surface is inherently inert and resists nonspecific adsorption; (ii) the surface contains uniform functional groups for the facile immobilization of protein molecules of interest; (iii) the linking chemistry allows the control of protein orientation and the local chemical environment favors the immobilized protein molecules to retain their native conformation; and (iv) the immobilization chemistry is highly specific and does not require prepurification of protein samples.

These criteria have not been met in past attempts based on passive adsorption of protein molecules into a polymer matrix. These approaches, derived from conventional methods, such as Western blot and ELISA, use gel pads, filter membranes or glass slides coated with polymer films for the immobilization of proteins [12–18]. The advantage of using a polymer matrix is the ease with which protein is immobilized, but there are also problems, including the inability to control protein conformation and orientation, the possibility of wash-off, as well as unknown diffusion kinetics or the inaccessibility of large target molecules into the polymer matrix.

Another class of methods generally relies on covalent bond formation between amine groups on protein molecules and other functional groups on a solid support. For example, MacBeath and Schreiber [19] demonstrated high-density protein arrays on glass slides through Schiff's base linkage formed from amine groups on proteins and aldehyde groups on silanized glass surfaces. Others immobilized proteins based on protein amine groups interacting with aldehyde groups [18], epoxide groups [20, 21], and carboxylic acid groups [22], or efficient leaving groups introduced by linkers [23, 24]. These approaches do not simultaneously meet all criteria set. In particular, the nonspecific adsorption problem is not addressed. After the immobilization of probe protein molecules, the surface is usually blocked with another protein, *e.g.*, BSA. However, these blocking molecules may affect the accessibility and conformation of immobilized proteins of interest. This is problematic, particularly for microarrays of small size protein molecules or peptides.

We believe the requirements set are mutually dependent and can be met simultaneously using a solid surface covered with an inert coating and using terminally tagged protein molecules. Surfaces that repel proteins, often referred to as "inert" or "nonfouling", have been studied extensively in the past [25–27]. The strong tendency for protein adsorption on most surfaces can be attributed to hydrophobic, ionic, and hydrogen bonding interactions with the solid surface. A result of these strong interactions is the increase in contact area (between a protein molecule and a solid surface), eventually leading to denaturation. Recently, Whitesides and coworkers [28] surveyed a large number of surface functional groups and concluded that the most extensively studied chemical entity, oligo or poly(ethylene glycol) [29], remains the most inert chemical group toward protein adsorption. A surface coated with high-density PEG functionality is an ideal substrate for protein immobilization because: (a) the intrinsic inertness of the surface permits minimal nonspecific adsorption; (b) the readily available alcohol functional groups on the surface of the PEG film can be easily activated to allow

selective and facile immobilization of protein molecules; (c) except for the activated surface sites where covalent bonds are formed between a protein molecule and the PEG film, all remaining surface sites are inert. This condition minimizes the contact area between a protein molecule and the solid surface and provides the ideal chemical environment for immobilized protein molecules to retain their native conformation. These properties, when coupled with genetically engineered tags, will also allow us to specifically attach protein molecules with controlled orientation.

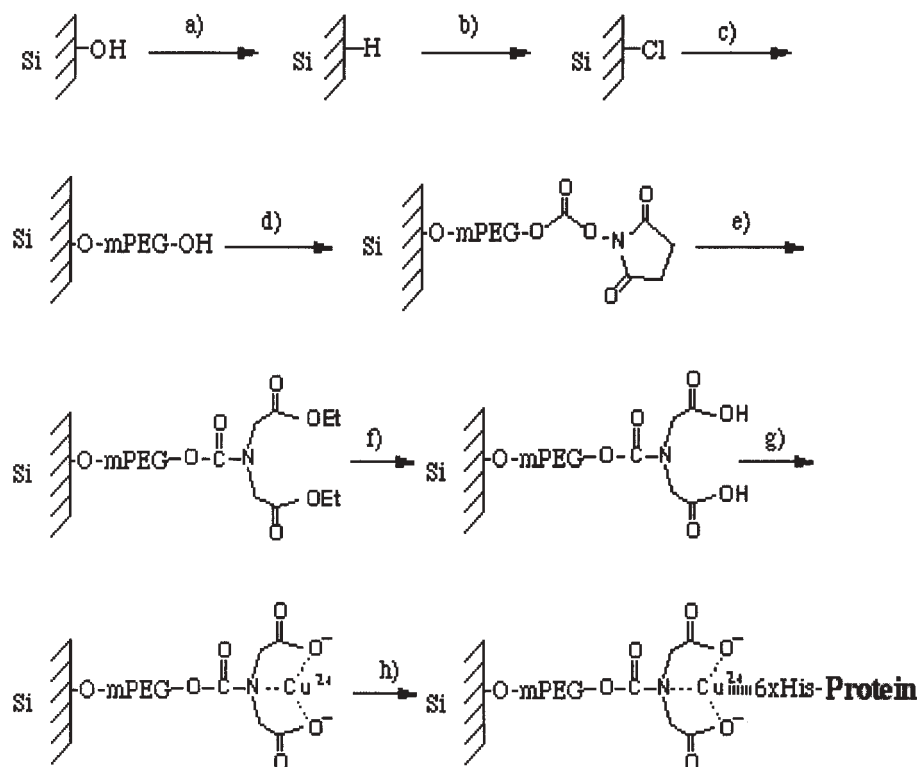
The advantage of using oligo(ethylene glycol) coatings for protein immobilization has been demonstrated most recently in a set of elegant papers by Mrksich and coworkers [30–32]. These authors successfully fabricated peptide, carbohydrate, and protein arrays on  $\omega$ -tri(ethylene glycol)-derivatized alkanethiol self-assembled monolayers (SAMs). The protein array is fabricated using a fusion protein containing the protein of interest and a capture protein which binds specifically to a capture ligand on the SAM before covalent bond formation. This method leads to oriented proteins with controllable density. A similar strategy was demonstrated earlier by Whitesides and coworkers [33], who used a mixed SAM of nitrilotriacetic acid (NTA) and tri-ethylene glycol terminated alkane thiols on gold for the immobilization of oriented protein molecules with polyhistidine tags. In all these examples, the nonfouling nature of the surface was critical to their success. Possible limitations of these approaches include the synthetic requirement for oligo(ethylene glycol) terminated alkanethiols and the instability of thiol SAMs due to the slow oxidation of the thiolate anchor. An alternative method to prepare PEG functionality for protein immobilization uses polylysine-grafted PEG [34].

In this report we show that a high-density, uniform, and robust PEG film can be formed on a Si(111) surface and this PEG-coated surface is ideally suited for protein immobilization. We demonstrate this for oriented protein molecules using polyhistidine tags and surface coordination chemistry. The advantages of the Si(111) substrate can be realized in its exceptional flatness on the atomic scale and its compatibility with microfabrication. The flatness of the Si(111) surface may allow nonconventional detection of probe-target interaction using, *e.g.*, atomic force microscopy (AFM), which is particularly attractive for nanoscale protein arrays. Previous research in our group has demonstrated that a high-density PEG brush can be formed on Si(111) based on the reaction of alcohol functional groups and Cl-terminated Si surface to form Si-O-C linkages [35]. This PEG/Si(111) surface has been demonstrated in the immobilization of oligonucleotides [36]. However, one disadvantage is the susceptibility of

the Si-O-C linkage to hydrolysis under strong acidic or basic conditions. We show here that this obstacle can be overcome by the use of a multi-arm PEG molecule (mPEG) which is capable of binding to the Si surface with multiple anchors, thus substantially increasing the stability of the grafted PEG film.

To achieve selective immobilization of protein molecules with controlled orientation, we borrow the well-established chemistry in immobilized metal ion affinity chromatography (IMAC) [37–40]. IMAC uses polyhistidine tags and chelated metal ions. In this method, a chelate group, e.g., iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA), is attached to a solid support. It can bind strongly to a metal ion such as  $\text{Ni}^{2+}$  or  $\text{Cu}^{2+}$  via three or four binding sites, leaving three or two vacant sites for coordination to imidazole groups of polyhistidine (His) units on the tagged protein molecule. The anchoring reaction between a poly-His tag and immobilized metal ion is fast and selective and may allow protein immobilization without prepurification. A poly-His tag can be engineered to either the C- or N-terminus of the protein molecule and it generally does not interfere with the structure or function of proteins and does not affect the secretion, compartmentalization, or folding of fusion proteins within cells [37–40]. Application of this chemistry for the immobilization of protein molecules on solid surfaces has been successfully demonstrated [33, 41–43].

Figure 1 outlines the synthetic scheme to immobilize 6x His-tagged protein molecules: (a) a native oxide terminated Si(111) surface is etched in  $\text{NH}_4\text{F}$  solution to give the H-terminated Si(111) surface; (b) the H-Si(111) surface reacts with  $\text{Cl}_2$  to give the activated Cl-Si(111) surface [35]; (c) the Cl-Si(111) surface reacts with an 8-arm PEG molecule for PEG immobilization with multiple Si-O-C anchors; (d) four out of the eight alcohol functional groups are accessible for coupling to disuccinimidyl carbonate (DSC); (e) diethyl iminodiacetate (DEIDA) is coupled to the surface through the displacement of the succinimidyl leaving group; (f) deprotection of the ethyl ester groups results in surface-attached chelating functionality, iminodiacetic acid (IDA); (g) the IDA group strongly binds divalent transition metal ions,  $\text{Cu}^{2+}$  ( $K_a \sim 10^{10.6}/\text{M}$ ) [44]. The  $\text{Cu}^{2+}$ -IDA system is chosen over the more common  $\text{Ni}^{2+}$ -nitrilotriacetic acid (NTA) combination because the former is known to have a higher binding affinity for histidine [44]; (h) the surface  $\text{Cu}^{2+}$ -IDA group selectively binds 6x His-tagged protein molecules. We characterize each step using a number of analytical techniques, including atomic force microscopy (AFM), multiple internal reflection-Fourier transform infrared (MIR-FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS), and fluorescence microscopy.



**Figure 1.** Reaction scheme for the immobilization of protein molecules on Si(111): (a) 40%  $\text{NH}_4\text{F}$ , 10 min, 25°C; (b)  $\text{Cl}_2$  gas, 20 min, 100°C; (c) mPEG, overnight, vacuum, 150°C; (d) DSC, DMAP, DMF, 6 h, 25°C; (e) DEIDA, DMAP, DMF, overnight, 25°C; (f) BTO, diethyl ether, 6 h, 25°C; (g)  $\text{CuSO}_4$ , ethanol, 20 min, 25°C; (h) 6x His-tagged protein incubation.

## 2 Materials and methods

### 2.1 Chemicals

All chemical reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) and Acros Organics (Morris Plains, NJ, USA) unless otherwise noted. Acetone and dichloromethane were HPLC grade. DMF was 99.9 + % biotech grade and used as received. Milli-Q water (18 M $\Omega$  cm) was used in all experiments. Multi-arm PEG (Ave.  $M_r$  = 9468, 8-arm,  $M_w/M_n$  = 1.08) was purchased from Shearwater (Huntsville, AL, USA) and used in a dry box. Ultra high purity Cl<sub>2</sub> gas (99.97%) was purchased from Scott Specialty Gases (Detroit, MI, USA). Si(111) wafers were n-type and had 0.005  $\Omega$  cm in resistivity (Wafernet, San Jose, CA, USA). Si(111) parallelogram plates attenuated total reflectance (ATR) plates designed for MIR-FTIR (45°, 50 × 10 × 1 mm<sup>3</sup>, 50 reflections, polished on both sides) were from Harrick Scientific (Ossining, NY, USA).

### 2.2 mPEG-coated Si(111)

The Si(111) wafer was cut into pieces of 1.5 × 1.5 cm<sup>2</sup> (chips) and cleaned in 3:1 solution of concentrated sulfuric acid and 30% hydrogen peroxide for 2 h at 100°C (Caution! This solution, called “piranha solution”, is a strong oxidant and reacts violently with organic materials.) After thorough rinsing with H<sub>2</sub>O, the cleaned Si samples were soaked in 40% deoxygenated NH<sub>4</sub>F solution for 10 min to prepare the hydrogen-terminated surface. The H-Si(111) samples were immediately moved to a glass reaction cell connected to a vacuum line, pumped down to  $\leq 10^{-3}$  torr, and exposed to Cl<sub>2</sub> (5 torr) for 20 min at 100°C. mPEG was a waxy solid at room temperature and a viscous liquid at 100°C. We applied a thin liquid film of mPEG by simply rubbing a piece of solid mPEG onto the Cl-Si(111) surface (held at 100°C) in an argon environment. The mPEG liquid covered sample was left overnight under vacuum at 150°C. The resulting mPEG-coated Si chip was rinsed with water three times and acetone two times to remove excess mPEG from the surface, and dried under a high-purity argon stream.

### 2.3 Succinimidyl derivatization of mPEG-Si(111)

mPEG-coated Si chips were immersed in the solution of 100 mM of disuccinimidyl carbonate (DSC) and 100 mM of dimethyl amino pyridine (DMAP) in DMF for 6 h at room temperature. Each chip was then rinsed with dichloromethane three times and dried under a high-purity argon stream.

### 2.4 Diethyl iminodiacetate (DEIDA) attachment

The succinimidyl derivatized mPEG-Si(111) chips were immersed in a solution of 200 mM of DEIDA and 100 mM of DMAP in DMF overnight at room temperature. The chips were then rinsed with dichloromethane three times and dried under a high-purity argon stream. The use of DEIDA without DMAP also gave the same result, albeit at a slower rate ( $\geq 24$  h).

### 2.5 Deprotection of surface attached DEIDA

The DEIDA-mPEG-Si(111) samples were immersed in a solution of 100 mM of bis(tributyltin) oxide (BBTO) in diethyl ether for 6 h at room temperature. The chips were then rinsed with diethyl ether three times, acidified with a dilute HCl solution (pH = 4) three times, rinsed again with acetone and dried under a high-purity argon stream.

### 2.6 Cu<sup>2+</sup>-IDA derivatized mPEG-coated Si(111)

The IDA-mPEG-Si(111) surfaces obtained from the deprotection of DEIDA functionalized samples were immersed in a solution of 2 mM of CuSO<sub>4</sub>·5H<sub>2</sub>O in ethanol for 20 min. The chips were then rinsed with water three times and dried under a high-purity argon stream.

### 2.7 Preparation of green fluorescent protein (GFP) and 6x His-GFP

cDNA of the coding region of eGFP with Flag-tag at the C-terminus was obtained by PCR using designed primers and then subcloned into pET 28c vector (Novagen, Madison, WI, USA). The resulting construct was transformed into bacteria host cells, BL21 (DE3). 2.5 mL of culture grown overnight at 37°C in the presence of kanamycin (30 mg/mL) was used to inoculate 50 mL of Luria broth media (with antibiotic). One mL of isopropyl-beta-D-thiogalactopyranoside (IPTG) was added when OD<sub>600</sub> of the media reached 0.5–0.7 to induce the expression. One mL of culture was saved for protein analysis on SDS-PAGE gel before IPTG induction. After 3–4 h, 1 mL of culture was saved and cells were harvested and resuspended in lysis buffer (pH 8) containing 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl and 10 mM imidazole (fit to pH 8 with NaOH). Lysate was sonicated (on ice) 6 × 10 s with 10 s pauses at 200–300 W after incubation with 1 mg/mL of lysozyme on ice for 30 min. High centrifugation was performed to remove insoluble material and debris. Supernatant (crude lysate) was saved for spotting. The majority of His-GFP was in the supernatant, indicating good solubility (negligible inclusion body). We did not determine the concentration of 6x His-GFP or GFP in the crude lysate solutions, which were used directly in the immobilization step below.

## 2.8 Immobilization of 6x His-GFP

The crude lysate solution of 6x His-GFP or GFP was spotted onto the  $\text{Cu}^{2+}$ -IDA-mPEG-Si(111) surface at 4°C by a micropipette. After incubation for 1 h at 4°C, the surface was rinsed with the washing buffer (50 mM of  $\text{NaH}_2\text{PO}_4$ , 300 mM of NaCl, 10 mM of imidazole, pH 8) and kept hydrated at 4°C for further imaging by fluorescence microscope. Note that imidazole was added to the washing buffer to remove weakly bound protein molecules due to coordination of single histidine residuals to surface  $\text{Cu}^{2+}$ .

## 2.9 Preparation of 6x His-STa IV

The procedure was similar to that described in Section 2.7 for His-tag GFP. Briefly, cDNAs of sulfotransferases IV (STa IV) with Flag-tag at the C-terminus were cloned into pET vectors (Novagen) and the resulting constructs were transformed into *Escherichia coli* host strains, such as BL21 DE3. Protein production was induced with IPTG. Crude lysates were prepared by resuspending bacterial pellet cells with lysis buffer, incubation with lysozyme (1 mg/mL) for 30 min, followed by sonication and high-speed centrifugation. Solubility of each protein was determined by SDS-PAGE gel. The amount of protein in crude lysates was normalized by SDS-PAGE gel to ensure equal amounts of protein were spotted on the array. To obtain purified proteins, we incubated crude lysate with Ni-NTA resin according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). After extensive washing, proteins were eluted from resin with high concentration imidazole. High concentration of imidazole was removed by dialysis. The concentration of purified 6x His-STa IV was determined to be 4.2  $\mu\text{g}/\mu\text{L}$ .

## 2.10 Preparation of 6x His-Sta IV arrays

A robotic spotter (Biorobotics, Cambridge, UK) was used to make arrays of 6x His-St IV. The His-St IV solution was aspirated from a well plate and spotted on the surfaces of interest ( $\text{Cu}^{2+}$ -IDA or  $\text{Cu}^{2+}$ -DEIDA surfaces). Each spot deposited was approximately 0.05 nL in volume and roughly 150–170  $\mu\text{m}$  in diameter with 400  $\mu\text{m}$  inter-spot spacing. The chip was then incubated for 2 h at room temperature in a humidity chamber, and washed with 1x PBS buffer (containing 0.05% Tween 20) three times (20 min each time) to remove any excess STa IV. Incubation of the chip with primary antibody was carried out overnight at 4°C, followed by washing with 1x PBS buffer (containing 0.05% Tween 20) three times (20 min each time) to remove any excess antibody. The surface was then incubated with Cy3-labeled secondary antibody for

2 h at room temperature under dark conditions. Washing was again achieved by immersion in 1x PBS buffer (containing 0.05% Tween 20) three times (20 min each time) to remove any excess secondary antibody. The sample was dried for fluorescence imaging.

## 2.11 X-ray photoelectron spectroscopy

All X-ray photoelectron (XP) spectra were taken on a Kratos XSAM 800 (Chestnut Ridge, NY, USA) with an Al  $\text{K}_{\alpha}$  X-ray anode operated at 266 W and a three-channel hemispherical electron energy analyzer. A background pressure of  $10^{-9}$  torr was maintained during XPS measurement. Path energy of 80 eV was used for survey scan and 40 eV for high resolution scan. The binding energy scale was referenced to the  $\text{Si}_{2p_{3/2}}$  peak from substrate Si (BE = 99.3 eV). The averaging time for each spectrum was approximately 30 min.

## 2.12 Multiple internal reflection-Fourier transform infrared spectroscopy

A modified MIDAC M2510-C FTIR spectrometer (MIDAC, Irvine, CA, USA) equipped with a liquid nitrogen-cooled mercury-cadmium-telluride detector was used. The spectrometer was purged by dry nitrogen during measurement. Both sides of the ATR Si crystal were coated and chemically derivatized. A clean ATR Si crystal after treatment with the "piranha solution" was used as background. All spectra were taken with an instrument resolution of 4  $\text{cm}^{-1}$ .

## 2.13 Fluorescence microscopy

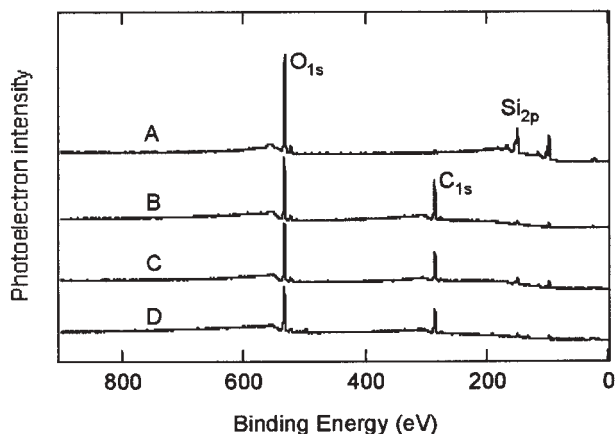
A Zeiss (Thornwood, NY, USA) fluorescence microscope fitted with a CCD camera and a 100 W mercury arc lamp was used to capture all fluorescence images with a 10 $\times$  objective lens. A typical image integration time was a few seconds.

# 3 Results and discussion

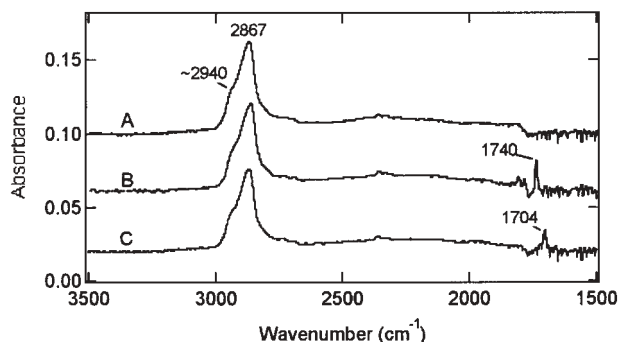
## 3.1 mPEG-coated Si(111)

H-Si(111) surface is prepared using the standard procedure of etching a native oxide terminated Si(111) in 40%  $\text{NH}_4\text{F}$  solution; this is known to give a monohydride covered surface with large atomically flat terraces and low defect density [45]. The H-terminated surface is converted to Cl-Si(111) by exposure to  $\text{Cl}_2$  gas at elevated temperature [35]. The Cl-Si(111) surface subsequently reacts with PEG for the grafting of the PEG monolayer [35].





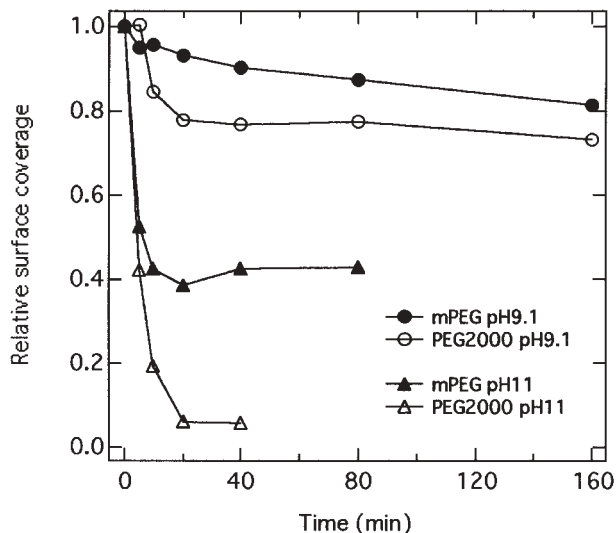
**Figure 4.** XPS survey spectra of (A) native oxide terminated Si(111); (B) mPEG-Si(111); (C) Cu<sup>2+</sup>-IDA-mPEG-Si(111); (D) 6x His GFP-Cu<sup>2+</sup>-IDA-mPEG-Si(111). All spectra are taken with a take-off angle of 60°.



**Figure 5.** MIR-FTIR spectra of (A) mPEG-Si(111); (B) succinimidyl derivatized mPEG-Si(111); and (C) DEIDA derivatized mPEG-Si(111).

growth condition, as shown by the absence of the carbonyl stretch peak around  $\sim 1700\text{ cm}^{-1}$ . Other spectra in this figure obtained after further derivatization will be discussed later.

We compare the stability of a linear (PEG2000,  $M_r = 2000$ ) and the mPEG grafted Si(111) surfaces. In the experiment, a PEG-coated surface is immersed in an aqueous solution at a particular pH. After removal from the solution following a certain incubation time, the sample is rinsed by water, dried under an Ar stream, and characterized by MIR-FTIR to determine surface coverage. While we found no significant loss of surface coverage for both linear PEG2000 and mPEG under neutral or acidic pH conditions, significant loss is seen for basic pH conditions. Figure 6 shows the results for pH = 9.1 and 11. The loss of surface coverage is clearly much slower for mPEG/Si(111) than that for PEG2000/Si(111). For example, at pH = 9.1 and 40 min incubation time, 23% of surface coverage is lost for PEG2000 while only 10% is lost



**Figure 6.** Relative surface coverage of PEG2000 (open symbols) and mPEG (solid symbols) grafted Si(111) as a function of incubation time in aqueous solutions at pH = 9.1 (circles) and 11.0, respectively. All surface coverages were determined by XPS.

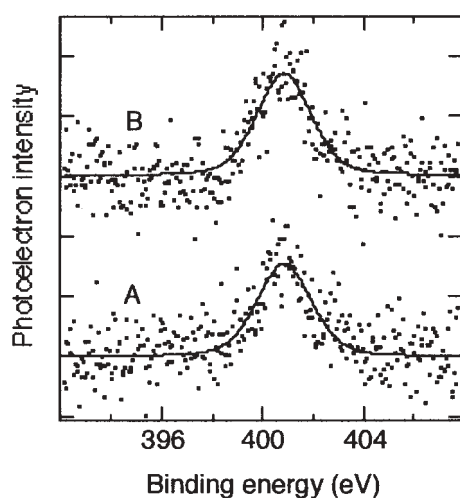
for mPEG. The difference is more significant at pH = 11: while almost all surface PEG2000 is lost after 20 min, the mPEG grafted surface stabilizes at a relative surface coverage of 40%. We have run the stability test for up to 300 min and observed no further decrease in surface mPEG coverage. There must be a distribution of grafted mPEG molecules with a different number of anchoring bonds to the Si surface. We believe those mPEG molecules stable against hydrolysis at pH = 11 must have the largest number of anchoring bonds.

### 3.2 Functionalization of the mPEG film

Functionalization and protein attachment on the mPEG-coated Si(111) surface, steps (d)–(h) in Fig. 1, are probed by MIR-FTIR, XPS, and fluorescence microscopy. In step (d),  $\omega$ -alcohol groups on the mPEG-coated Si(111) surface are converted to succinimidyl leaving groups using disuccinimidyl carbonate (DSC), which is known as a homobifunctional cross-linker for the activation of the hydroxyl group [46]. The reactivity of the second leaving group is less than that of the first leaving group. After attachment reaction to the mPEG surface, the conversion of each bilinker to a bridge surface species is unlikely. Note that this reaction is known to be efficient and, in the presence of excess DSC, we expect each accessible  $\omega$ -alcohol group on the mPEG-coated Si(111) surface to be converted. As shown below, quantitative analysis reveals that four out of eight  $\omega$ -alcohol groups on each

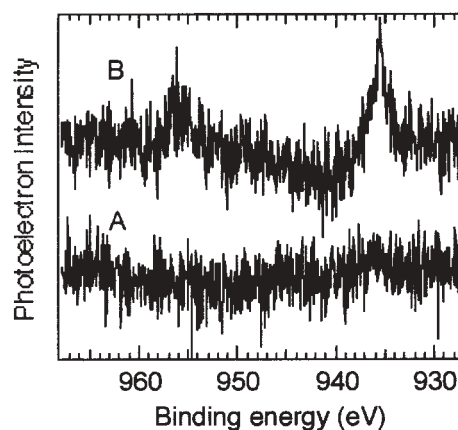
mPEG molecule react with DSC. The remaining four  $\omega$ -alcohol groups are believed to be involved in bonding to the surface.

The presence of the succinimidyl group on the surface is evidenced by the appearance of the characteristic carbonyl stretch peak at  $1740\text{ cm}^{-1}$  [47]. After further reaction with DEIDA, the carbonyl peak attributed to succinimidyl disappeared and is replaced by a new carbonyl peak at  $1704\text{ cm}^{-1}$ , which is assigned to the DEIDA functionality. The success of these two reaction steps is also shown by the  $\text{N}_{1s}$  signal in XPS, as shown in Fig. 7 for both DSC derivatized or DEIDA attached mPEG-Si(111) surfaces. The  $\text{N}_{1s}$  peak areas (and thus atomic concentrations) of these two surfaces are the same within experimental uncertainty, as expected from stoichiometry.



**Figure 7.** XPS spectra in the  $\text{N}_{1s}$  region for (A) DSC derivatized and (B) DEIDA attached mPEG-Si(111) surfaces. The dots are data points and the solid lines are fits to a single Gaussian-Lorentzian function. Both spectra are taken with a take-off angle of  $0^\circ$ .

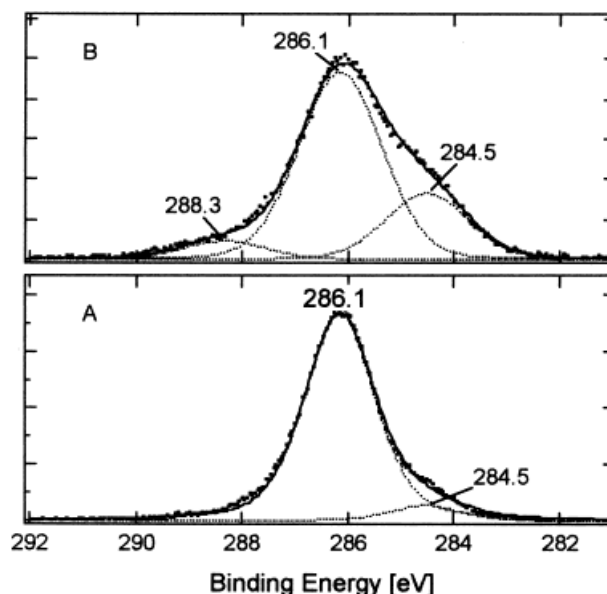
To convert surface DEIDA to the chelating IDA group, we need to deprotect the two ethyl groups in each DEIDA. The commonly used deprotection method based on aqueous solutions at very high pH ( $\geq 11$ ) is not suitable because of the damage to the Si-O-C anchoring bonds. Instead, we chose deprotection reaction under mild conditions, particularly, the use of bis(tributyltin) oxide (BBTO), which is known as a highly chemo-selective cleavage reagent to yield free carboxylic acid groups [49]. This alternative approach is carried out in aprotic solvent at neutral condition. The success of this deprotection reaction is verified indirectly based on the capability of the surface to bind  $\text{Cu}^{2+}$ . Figure 8 shows XPS in the  $\text{Cu}_{2p}$  region taken after  $\text{Cu}^{2+}$  adsorption on the DEIDA-mPEG-Si(111) surface before and after the BBTO depro-



**Figure 8.** XPS spectra in the  $\text{Cu}_{2p}$  region taken after  $\text{Cu}^{2+}$  adsorption on (A) DEIDA-mPEG-Si(111) and (B) IDA-mPEG-Si(111) surfaces. Note that  $\text{Cu}^{2+}$  is reduced to  $\text{Cu}^{1+}$  by secondary electrons during XPS measurement [48]. Both spectra are taken with a take-off angle of  $0^\circ$ .

tection step.  $\text{Cu}^{2+}$  only absorbs on the surface after treatment by BBTO. This can be attributed to generation of the IDA chelating group which strongly binds  $\text{Cu}^{2+}$ .

The extent of surface attachment reaction can be calculated quantitatively from high-resolution XPS of the  $\text{C}_{1s}$  region. Spectrum (A) in Fig. 9 shows  $\text{C}_{1s}$  XP spectrum of



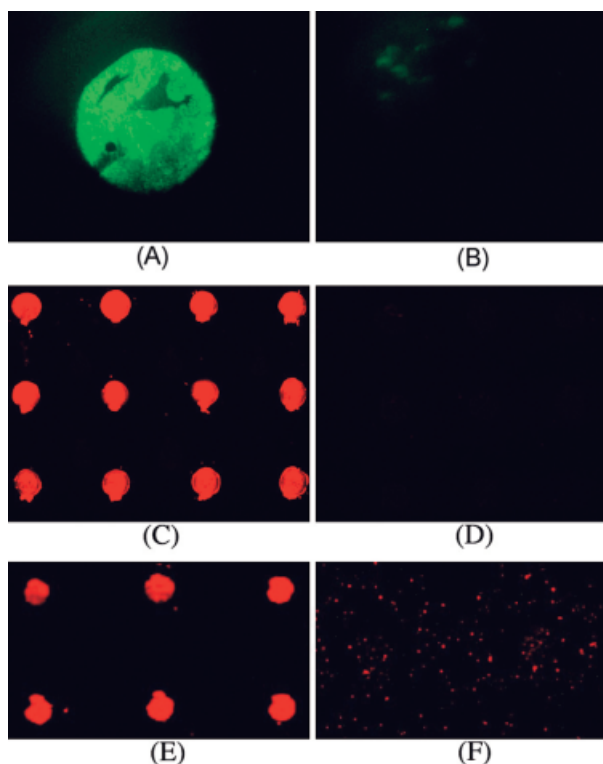
**Figure 9.** XPS spectra in the  $\text{C}_{1s}$  region of (A) mPEG-Si(111) and (B)  $\text{Cu}^{2+}$ -IDA-mPEG-Si(111). The dots are experimental data; the solid line on each spectrum is the overall fit and dashed lines are individual components in the fit. These individual fitting components are of the same convoluted Gaussian-Lorentzian peak shape and peak width. Both spectra are taken with a take-off angle of  $60^\circ$ .

the mPEG-Si(111) surface. The spectrum is dominated by a peak at electron binding energy (BE) of 286.1 eV, which is assigned to the immobilized PEG molecule [50]. The small peak at 284.5 eV is due to hydrocarbon contamination. After steps (d–g) in Fig. 1, XPS of the resulting  $\text{Cu}^{2+}$ -IDA-mPEG-Si(111) shows a main peak at 286.1 eV due to the PEG molecule and two additional peaks at BE = 288.3 and 284.5 eV, respectively. The peak at 288.3 eV is at the expected BE position of carboxylate groups [50] in the surface attached IDA. The peak at 284.5 eV can be attributed to increased hydrocarbon contamination resulting from the multiple processing steps. The two  $\text{CH}_2$  units within each IDA group also contribute to this low BE peak. Based on the relative intensities of the carboxylate peak (288.3 eV) and the PEG peak (286.1 eV), we obtain a relative concentration of four IDA groups for each surface mPEG molecule (see below).

### 3.3 Immobilization of 6x His-tagged protein molecules

To test the surface in binding 6x His tagged protein molecules with high specificity, we chose GFP and sulfotransferase (ST). GFP is selected because of its robustness and the presence of an intrinsic chromophore which allows us to easily verify that the immobilized protein retains its native conformation [51, 52]. The sulfotransferases refer to an entire family of enzymes of detoxication that catalyzes the transfer of the sulfuryl group,  $\text{SO}_3^-$ , from adenosine 3'-phosphate 5'-phosphosulfate (PAPS) to a wide range of xenobiotics, such as phenols, alcohols and amines, etc. This model system is chosen because the mechanism and substrate specificity for this family of enzymes have been well characterized [53–55]; they are known to have broad substrate specificity and are ideal candidates for the demonstration of the power of protein microarray in high-throughput analysis of a wide range of protein-ligand interactions. Previous studies have also indicated that sulfotransferases can be expressed in *E. coli* as soluble forms that are active with xenobiotics [56–58].

Crude lysates of GFP with or without a 6x His tag are manually spotted onto the  $\text{Cu}^{2+}$ -IDA-mPEG-Si(111) surface. After extensive washing with imidazole containing buffer solution, each surface is maintained in a hydrated state and imaged by fluorescence microscope. The strong green fluorescence on the 6x His-GFP-treated sample (Fig. 10A) clearly shows the successful immobilization of the protein molecule. In contrast, the sample treated with GFP (without 6x His tag, Fig. 10B) shows nearly no fluorescence signal (not significantly above background level without GFP), indicating the absence of protein immobilization. These experiments establish



**Figure 10.** Fluorescence microscope images of: (A) 6x His-GFP and (B) GFP on the  $\text{Cu}^{2+}$ -IDA-mPEG-Si surface; (C) purified 6x His-ST IV on the  $\text{Cu}^{2+}$ -IDA-mPEG-Si surface; and (D) background test which involves spotting 6x His-ST onto a DEIDA-mPEG-Si surface after pretreatment with  $\text{CuSO}_4$  solution; (E) crude lysate containing 6x His-ST IV on the  $\text{Cu}^{2+}$ -IDA-mPEG-Si surface; and (F) background test which involves spotting of crude lysate containing 6x His-ST IV onto the DEIDA-mPEG-Si surface after pretreatment with  $\text{CuSO}_4$  solution. Protein detection in (C–F) is achieved by incubation with primary antibody against ST IV, followed by a Cy3-labeled secondary antibody. The diameters of all spots are  $\sim 1$  mm.

that immobilization of the GFP molecule to the  $\text{Cu}^{2+}$ -IDA-mPEG-Si(111) surface is specific through the 6x His tag. Nonspecific adsorption is negligible on this otherwise inert surface. Because the 6x His tag is engineered only to the C-terminus of the protein molecule, the surface immobilized GFP should possess controlled orientation. Another key result is that the difficult and time-consuming step of protein purification is not needed for such a highly specific immobilization reaction.

The success of this specific immobilization reaction is also demonstrated for the model enzyme, sulfotransferase IV (ST IV). In the first experiment, arrays of purified 6x His-tagged STa-IV are spotted onto the surface by a robotic spotter. The surface is subsequently incubated with primary antibody and detected with a Cy3-labeled

secondary antibody. Figure 10C shows fluorescence image of a  $3 \times 4$  array of 6x His-ST IV immobilized on the  $\text{Cu}^{2+}$ -IDA-mPEG-Si(111) surface. As a background test, we show that there is no protein adsorption when  $\text{Cu}^{2+}$  is absent. In such a background test, a DEIDA-mPEG-Si(111) surface (without the deprotection of ethyl groups) is soaked in  $\text{CuSO}_4$  solution and washed. As shown previously (Fig. 7), there is no  $\text{Cu}^{2+}$  adsorption on this surface. Purified 6x His-ST IV is then spotted and detected under identical conditions as for the  $\text{Cu}^{2+}$ -IDA-mPEG-Si(111) surface in Fig. 10C. Fluorescence image of the resulting sample is shown in Fig. 10D. There is no detectable ST IV on the surface.

In the second experiment, we used crude lysate and repeated the experiment under otherwise identical conditions as in Figs. 10C and D. Figure 10E shows the successful immobilization of 6x His-tagged STa-IV from the crude lysate sample on the  $\text{Cu}^{2+}$ -IDA-mPEG-Si(111) surface, but not in the absence of surface  $\text{Cu}^{2+}$  (Fig. 10F). Compared to results in Figs. 10C and D, the use of unpurified protein sample leads to a slight increase in background signal, particularly dust-like particulates in the fluorescence image (Fig. 10F).

### 3.4 Quantitative determination of surface coverages

The coverages of all chemical species on the surface are determined by XPS and summarized in Table 1. The average thickness of the mPEG film on the Si(111) surface can be obtained from the attenuation of substrate  $\text{Si}_{2p}$  signal based on:

$$\frac{I}{I_0} = \exp\left(-\frac{L}{\lambda \cos\theta}\right) \quad (1)$$

where  $I_0$  and  $I$  are the intensity of  $\text{Si}_{2p}$  before and after mPEG coating, respectively;  $L$  is the thickness of the monolayer;  $\theta$  is the angle of photoelectron detection (from surface normal); and  $\lambda = 3.65$  nm, the attenuation length of  $\text{Si}_{2p}$  photoelectron in PEG [35]. The hydrogen-

terminated Si(111) is used for  $I_0$ , the reference  $\text{Si}_{2p}$  intensity. This calibration gives mPEG film thickness of  $L = 5.2$  nm. If we assume the density of bulk solid mPEG ( $d = 0.56$  g/cm<sup>3</sup>), this thickness is converted to a molecular grafting density of  $1.9 \times 10^{13}$  molecules/cm<sup>2</sup>. After multiple steps of surface functionalization (steps d–g in Fig. 1), we see a slight decrease in the  $\text{C}_{1s}/\text{Si}_{2p}$  peak intensity ratio (see spectrum C in Fig. 4), indicating the loss of some PEG molecules from the surface. Based on the change in  $\text{C}_{1s}$  signal from PEG and the change in  $\text{Si}_{2p}$  intensity from the substrate, we calculate a final mPEG film thickness of  $L = 4.6$  nm for the  $\text{Cu}^{2+}$ -IDA-mPEG-Si(111) surface, corresponding to  $1.7 \times 10^{13}$  mPEG molecules/cm<sup>2</sup>. Thus, there is a 12% loss of surface grafted mPEG molecules following the four processing steps. We expect that the number of anchoring bonds per mPEG molecule to vary in the monolayer and those with fewer anchoring bonds are more likely to desorb from the surface during various solution treatments in steps (d–g) of Fig. 1.

The number of DEIDA or IDA groups attached to each surface mPEG molecule can be obtained from  $\text{C}_{1s}$  intensity ratio of the carboxylate peak (BE = 288.3 eV) and the PEG peak (BE = 286.1 eV) in Fig. 8B. Considering the attenuation factor in a homogeneous monolayer with a thickness ( $L$ ), the total  $\text{C}_{1s}$  photoelectron intensity ( $I_{\text{PEG}}$ ) from the mPEG film can be expressed as:

$$\begin{aligned} I_{\text{PEG}} &= \alpha(\text{C}_{1s}) \frac{[\text{PEG}]}{L} N_{\text{PEG}} \int_0^L \exp\left(-\frac{L-z}{\lambda \cos\theta}\right) dz = \\ &= \alpha(\text{C}_{1s}) [\text{PEG}] N_{\text{PEG}} \frac{\lambda \cos\theta}{L} \left(1 - \exp\left(-\frac{L}{\lambda \cos\theta}\right)\right) \quad (2) \end{aligned}$$

where  $\alpha(\text{C}_{1s})$  is an instrument specific sensitivity factor for the  $\text{C}_{1s}$  photoelectron;  $[\text{PEG}]$  is the surface concentration of mPEG ( $= 1.7 \times 10^{13}$  mPEG molecules/cm<sup>2</sup>);  $N_{\text{PEG}}$  ( $= 426$ ) is the total number of carbon atoms in each PEG molecule;  $z$  is the distance from the silicon surface and  $L$  ( $= 4.6$  nm) is the film thickness;  $\lambda$  ( $= 3.54$  nm) is the attenuation length of  $\text{C}_{1s}$  photoelectron in the PEG film [59].

**Table 1.** Concentrations (molecules/cm<sup>2</sup>) of surface species

Surface species	mPEG <sup>a)</sup>	DEIDA/IDA	IDA- $\text{Cu}^{2+}$	GFP
Coverage (cm <sup>-2</sup> )	$1.7 (1.9) \times 10^{13}$	$6.8 \times 10^{13}$	$2.7 \times 10^{13}$	$\sim 2 \times 10^{13}$

a) Number in parenthesis corresponds to the starting coverage of mPEG molecules before further derivatization.

The  $C_{1s}$  signal from carboxylate carbon in DEIDA or IDA groups on the surface of the PEG film can be taken as unattenuated:

$$I_{IDA} = \alpha(C_{1s})[IDA]N_{IDA} \quad (3)$$

where  $[IDA]$  is the surface concentration of DEIDA and IDA groups and  $N_{IDA}$  ( $= 3$ ) is the number of carboxylate carbon atoms in each attached DEIDA or IDA group.

Based on the experimental  $I_{IDA}/I_{PEG}$  ratio in Fig. 8B and Eqs. 2 and 3, we obtain a surface concentration ratio of  $[IDA\text{-mPEG}]/[mPEG] = 4.12$ . Thus, approximately four of the eight branches on each mPEG molecule are derivatized with a DEIDA/IDA group.

We believe the strong affinity of IDA for  $Cu^{2+}$  guarantees that each surface IDA group bonds one  $Cu^{2+}$  ion after step (g) (Fig. 1). Based on the XPS sensitivity factors [50] for  $Cu_{2p}$  and  $N_{1s}$  and the experimental intensity ratio for these two transitions from the surface after step (g), we calculate a surface atomic concentration ratio of  $[Cu]/[N] = 0.40$ , lower than the expected ratio of unity for a fully IDA derivatized surface. We attribute this to the low yield of the deprotection reaction, step (f) in Fig. 1. Because of the mild reaction condition, only 40% of surface DEIDA groups are deprotected to give IDA. This is in agreement with the reported yield of the deprotection reaction [49].

We can also estimate the coverage of protein molecules after the immobilization reaction, step (h) in Fig. 1. XPS analysis of the 6x His-GFP immobilized surface (spectrum D in Fig. 4) shows the further attenuation of substrate  $Si_{2p}$  signal compared to the  $Cu^{2+}$ -IDA-mPEG-Si(111) surface (spectrum C in Fig. 4). Based on this attenuation and the same electron escape depth used for the calculation of PEG film thickness, we calculate a GFP film thickness of 4.9 nm. Interestingly, this estimated thickness is close to the height ( $\sim 4.2$  nm) of a GFP molecule along the axis of the barrel-like structure. In fact, if we assume that these GFP barrels (diameter  $\sim 2.4$  nm) form a close-packed monolayer on the surface, we arrive at a surface coverage of  $\sim 2 \times 10^{13}$  molecules/cm<sup>2</sup>, which is in good agreement with the surface coverage of chelated  $Cu^{2+}$ .

#### 4 Concluding remarks

We demonstrate a high-density PEG-coated Si(111) surface as an ideal substrate for the selective immobilization of polyhistidine-tagged protein molecules. Because the poly-His tag can be engineered to either the C- or the N-terminus of a protein molecule, such a strategy leads to immobilized protein molecules with controlled orientation on the surface. The high-density PEG-coated silicon

surface is obtained from the reaction of a multi(8)-arm PEG molecule with a chlorine terminated Si(111) surface to form C-O-Si linkages; the presence of multiple anchoring bonds to the surface substantially improves the stability of the mPEG monolayer. Four out of the eight arms on each immobilized mPEG molecule are accessible for linking to IDA groups for the binding of  $Cu^{2+}$  ions. The resulting  $Cu^{2+}$ -IDA-mPEG-Si(111) surface is shown to specifically bind 6x histidine-tagged protein molecules, including green fluorescent protein and sulfotransferase IV, without the need of prepurification. In the case of 6x His-GFP, this immobilization strategy can lead to a closely packed monolayer of protein molecules. Background tests show that the surface retains its inertness towards nonspecific protein adsorption in the absence of either a poly-His tag on the protein molecule or metal ions on the surface. Both the inertness of the surrounding chemical and the controlled orientation should contribute to an ideal environment for the immobilized protein molecule to retain its native conformation and reactivity. Experiments are underway to establish the kinetics of substrate binding and enzyme activity for surface immobilized sulfotransferase IV.

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