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SHORT COMMUNICATION

Enzymatic activity on a chip: The critical role of protein orientation

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We compare the catalytic activities of enzymes immobilized on silicon surfaces with and without orientation. While oriented sulfotransferases selectively immobilized on an otherwise zero-background surface *via* 6×His tags faithfully reflect activities of solution phase enzymes, those with random orientation on the surface do not. This finding demonstrates that controlling the orientation of immobilized protein molecules and designing an ideal local chemical environment on the solid surface are both essential if protein microarrays are to be used as quantitative tools in biomedical research.

Keywords:
Immobilized metal ion affinity chromatography / Protein microarray / Protein orientation / Sulfotransferase

One of the most exciting tools in proteomics is the protein microarray technology, in which a large number of proteins or peptides are immobilized on a solid substrate for the high-throughput analysis of biochemical properties and biological activities [1]. Compared to its counter part in genomics (*i.e.*, DNA microarrays), there are two inherent difficulties associated with protein immobilization: (i) Background. Proteins tend to adsorb nonspecifically to solid substrates, leading to not only the possibility of denaturation but also background problems during assays; (ii) conformation and orientation. Because proteins have complex structures and activities, the immobilization chemistry has to be such that it preserves a protein in native state and with optimal orientation for protein-target interaction. Past attempts on protein immobilization for microarrays have mainly used nonspecific adsorption [2–6] or covalent bond formation between readily available functional groups (*e.g.*, -NH_2) on protein molecules and complimentary coupling groups (*e.g.*, aldehyde or epoxide) on the solid surfaces [7, 8]. A major concern with both approaches is that the protein molecules are randomly oriented on the surfaces. As a result, the active sites of a substantial population of immobilized protein molecules are not accessible to targets in the solution phase. The non-specific nature of these approaches inevitably requires the use of purified protein samples. In addition, there is a possibility of denaturing when the interaction between randomly immobilized protein and the surface is too strong.

The importance of selective immobilization of protein molecules with orientational control has been recognized most recently [9]. Demonstrated strategies include the use of fusion proteins [10], immobilized protein A or G which binds to the Fc portion of antibodies [11, 12], mRNA-protein hybrids [13], chemical modifications based on biotin-streptavidin interaction [14] or Staudinger ligation reaction [15]. Proteins or antibodies with uniform and controlled orientation have been shown to possess higher activity than those of random orientation [14, 15]. Disadvantages of chemical modification include limited applicability and the requirement for purified samples.

We have adopted the strategy for oriented protein microarrays using recombinant poly-histidine (poly-His) tags and surface-chelated metal ions on an otherwise "zero"-background surface [16]. This strategy originates from immobi-

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Abbreviations: AST IV, phenol sulfotransferase IV; **IMAC**, immobilized metal ion affinity chromatography; **PAPS**, 3′-phosphate 5′-phosphosulfate

lized metal ion affinity chromatography (IMAC) [17] and has been applied to protein immobilization [18–21]. There are a number of advantages of developing this method into a general strategy for the fabrication of protein microarrays. The generation of a poly-His tag to either the *C*-terminus or *N*-terminus is perhaps the most commonly used method in recombinant protein technology. Unlike other fusion protein strategies, the poly-His tag approach for purification can be applied not only to proteins in native states, but also to those under denature conditions or to small peptides. When applied to protein microarray technology, this strategy effectively combines the steps of purification and immobilization. The anchoring bond is highly stable and reversibility occurs only in the presence of a high concentration of competing ligands, such as imidazole. To take advantage of the high specificity of binding between a poly-His tag and chelated metal ions, the surface must resist the nonspecific adsorption of all other protein molecules lacking the poly-His tag. For this purpose, we have developed methods for the grafting of high-density poly(ethylene) glycol (PEG) films on silicon surfaces. The intrinsic inertness of the PEG functionality permits minimal nonspecific adsorption of proteins, while the readily available alcohol functional groups on the surface of the PEG film can be easily activated for metal ion adsorption. Except for the poly-His tag on the *N*- or *C*-terminus, each immobilized protein molecule stays away from and minimizes its interaction with the surface due to the repulsive nature of the PEG environment. As a result, there is minimal disturbance to the native conformation of the protein. With the above success in immobilization chemistry [16], we are now able to ask two critical questions: (i) Are the immobilized enzymes in native states and accessible to targets in the solution phase? (ii) Are the oriented proteins truly advantageous over randomly oriented proteins in terms of activity?

We address these two questions in the present study using the model system of phenol sulfotranferase (AST IV). The sulfotransferases refer to an entire family of enzymes of detoxication that catalyzes the transfer of the sulfuryl group, 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. We compare the enzymatic activities of the 6×His tagged AST IV in the solution phase with those immobilized with controlled orientation on the Cu^{2+}-IDA-mPEG-Si(111) surface or with random orientations on surfaces. To achieve immobilization with random orientation, we prepare two types of surfaces. The first surface is an epoxy-functionalized silane monolayer on native oxide-terminated Si(111) from 3-glycidoxypropyl trimethoxysilane (Fig. 1A); the second surface is the multiarm PEG (mPEG) monolayer-covered Si(111) activated by disuccinimidyl carbonate (DSC) (Fig. 1B). Both surfaces are reactive towards -NH_2 functional groups on protein molecules for covalent attachment. Since there are many lysine

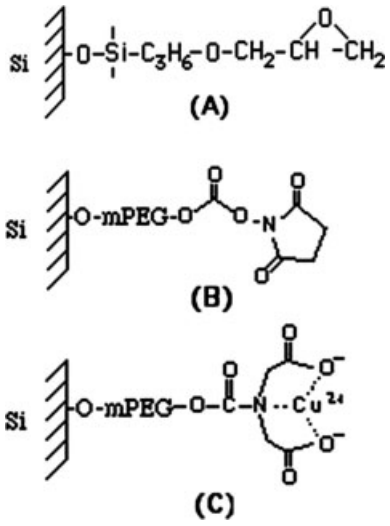

Figure 1. The three coatings for the immobilization of (A, B) randomly distributed and (C) oriented proteins on the silicon surface.

residuals on the protein molecules, each sulfotransferase can be immobilized with a variety of orientations on these two surfaces.

Details on the preparation of surfaces (B) and (C) in Fig. 1 have been presented earlier [16]. Surface A was prepared by the evaporation of 3-glycidoxypropyl trimethoxysilane onto the freshly cleaned native oxide-terminated Si(111), followed by extensive rinsing with dichloromethane. Sulfotranferase IV with a 6×His tag at the *C*-terminus (6×His-AST IV) was obtained by standard recombinant technology [16]. We used purified 6×His-AST IV solution with a protein concentration of 18.1 μg/μL in all experiments. In immunoassays, a robotic spotter (Biorobotics, Cambridge, UK) was used to make arrays of 6×His-AST IV on each surface in Fig. 1. Each spot deposited was approximately 0.05 nL in volume and roughly 150–170 μm in diameter. Each chip was then incubated for 2 h at room temperature in a humidity chamber, and washed with 1× PBS buffer (containing 0.05% Tween 20) three times (15 min each) to remove any excess 6×His-AST IV. Incubation of the chip with primary antibody (1:400 dilution) [22] was carried out overnight at 4°C, followed by washing with 1× PBS buffer three times (15 minute each) to remove any excess antibody. The surface was then incubated with Cy3-labeled secondary antibody (1:100 dilution) for 1 at room temperature in dark. Washing was again achieved by immersion in 1× PBS buffer three times (15 min each) to remove any excess secondary antibody. The sample was dried for fluorescence imaging. A Zeiss fluorescence microscope fitted with a CCD camera and a 100 W mercury arc lamp was used to capture all fluorescence images with a 10× objective lens.

In kinetic measurements, we immobilized protein molecules uniformly on each silicon sample with dimension of 10 × 35 mm^2. 20 μL 6×His-AST IV solution was used to completely cover the surface of each silicon sample. Each chip was then incubated for 2 h at 4°C in a humidity chamber, and then soaked in excess 1× PBS buffer for 30 min to

remove any excess 6×His-AST IV. The silicon sample with immobilized AST was transferred to the sample cell. The reaction medium contained PAPS (0.4 mM), β-mercaptoethanol (4.8 mM) in trispropane (53 mM) buffer at pH 7. After recording a baseline fluorescence intensity, the fluorescent substrate, resorufin (39 mM in DMSO), was added and the decay of fluorescence intensity was recorded as a function of time to quantify enzyme activity. Fluorescence measurement was carried out on a Quantamaster QM-2000-7 spectrometer (Photon Technology International, Lawrenceville, NJ, USA). The excitation and emission wavelength was set at 540 nm and 585 nm, respectively.

Figure 2 shows results from immunoassay of 6×His-AST IV immobilized on the three surfaces in Fig. 1. The two surfaces (A and B) with randomly immobilized 6×His-AST IV show nearly identical fluorescence intensities, indicating similar surface protein concentrations. On the other hand, oriented 6×His-AST IV on Cu-IDA-mPEG-Si (C) gives a 70% more fluorescence intensity. This higher fluorescence intensity from the oriented array may not correspond to a higher surface protein concentration; instead, we believe that an immobilized protein molecule with controlled orientation is more accessible to the antibody. This is not surprising because, for immobilized proteins with random orientations, the binding sites of some of the protein molecules on the surface are blocked by the solid substrate and inaccessible to antibodies.

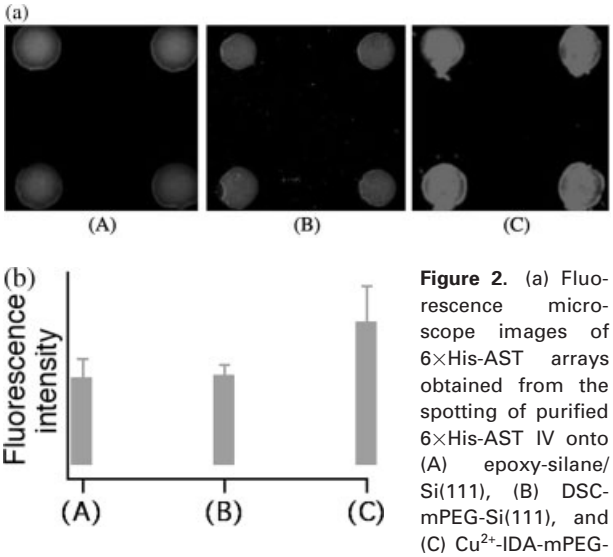

Figure 2. (a) Fluorescence microscope images of 6×His-AST arrays obtained from the spotting of purified 6×His-AST IV onto (A) epoxy-silane/Si(111), (B) DSC-mPEG-Si(111), and (C) Cu^{2+}-IDA-mPEG-Si(111) surfaces. Protein detection is achieved by incubation with primary antibody against AST IV, followed by Cy3-labeled secondary antibody. (b) Fluorescence intensities from the three surfaces shown in (a). The error bars are obtained from the statistical analysis of various spots on the same sample.

We estimate the surface concentration of immobilized AST IV as follows. On the Cu^{2+}-IDA-mPEG-Si(111) surface, the Cu^{2+} concentration is 2.7×10^{13} cm^{-2} [16], which represents the upper limit of surface concentration for 6×His-AST IV if every surface Cu^{2+} site is involved in binding to a 6×His-tagged protein molecule. Indeed, we estimate from X-ray photoelectron spectroscopy (XPS) that the surface concentration of immobilized protein molecules is $\sim 2 \times 10^{13}$ cm^{-2}, close to this upper limit [16]. This corresponded to a nearly close-packed monolayer of protein molecules. Note that the actual unit area for each immobilized molecule is higher, due to the morphology of the mPEG film (root-mean-square roughness = 4 nm) [16]. Considering that the immunoassay of randomly oriented 6×His-AST IV on epoxy or succinimidyl carbonate functionalized surfaces showed 60% of the fluorescence intensity of oriented 6×His-AST IV, and substantial proportions of these protein molecules should be inaccessible to antibodies, we believe that the actual surface concentrations of randomly oriented protein molecules are similar to that of oriented 6×His-AST IV on Cu^{2+}-IDA-mPEG-Si(111).

More dramatic differences between oriented and random proteins are seen in enzymatic activity. We characterize enzyme kinetics using the method of Beckmann [23] who showed that phenol sulfotransferases can catalyze the sulfation of a fluorescent compound, resorufin, to its non-fluorescent derivative. Thus, we can simply follow the catalytic reaction in the time domain by recording fluorescence decay of the reactant. The assay used PAPS as a sulfuryl group donor and the fluorescent substrate, resorufin, as a sulfuryl group acceptor. Figure 3a shows the fluorescence decay for the sulfuryl transfer reaction catalyzed by (A) randomly oriented 6×His-AST IV immobilized on the epoxylsilane/Si(111) surface, (B) randomly oriented 6×His-AST

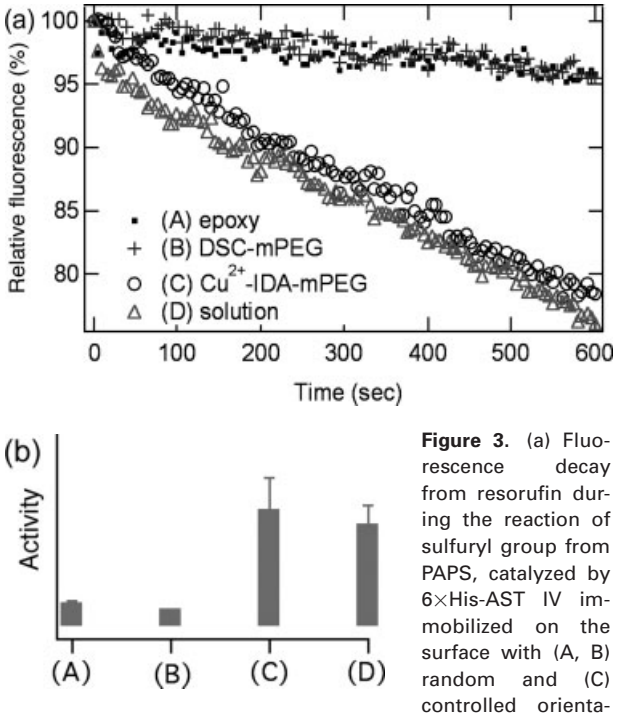

Figure 3. (a) Fluorescence decay from resorufin during the reaction of sulfuryl group from PAPS, catalyzed by 6×His-AST IV immobilized on the surface with (A, B) random and (C) controlled orientation, or (D) in the solution phase. (b) Initial reaction rates for (A)–(D). The error bars are from the statistical analysis of repeated experiments.

IV immobilized on DSC activated mPEG/Si(111) surface, (C) oriented 6×His-AST IV on the Cu^{2+}-IDA-mPEG-Si(111) surface, and (D) 6×His-AST IV in the solution phase with the amount of protein molecules equal to that on each chip (surface). We take the initial velocity (negative slope) as a measure of enzymatic activity, as summarized in Fig. 3b. The enzymatic activities of randomly oriented protein molecules are the same for the two surfaces with different immobilization chemistry (A and B); they are 5–6 times lower than that of the oriented sample. Within experimental uncertainty, the enzymatic activity of oriented AST IV on the surface (C) is the same as that of free enzyme molecules in the solution phase (D).

The above results clearly establish the critical importance of controlling the orientation of immobilized molecules in protein microarray technology. While oriented protein molecules selectively immobilized on the PEG surface *via* the 6×His tag faithfully reflect activities of solution phase proteins, those with random orientation on the surface do not. This observation can be understood because the active sites on certain population of randomly oriented protein molecules on the surface are not accessible. The possible presence of multiple covalent bonds between a randomly oriented protein molecule and the solid surface may also affect its conformation. We conclude that controlling the orientation of immobilized protein molecules and designing an ideal local chemical environment on the solid surface are both essential if protein microarrays are to be used as quantitative tools in biomedical research.

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