ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Directed Self-Immobilization of Alkaline Phosphatase on Micro-Patterned Substrates Via Genetically Fused Metal-Binding Peptide

Turgay Kacar,[1,2] Melvin T. Zin,[1] Christopher So,[1] Brandon Wilson,[1] Hong Ma,[1] Nevin Gul-Karaguler,[2] Alex K.-Y. Jen,[1,3] Mehmet Sarikaya,[1,4] Candan Tamerler[1,2]

[1]Department of Materials Science and Engineering, University of Washington, Seattle, Washington 98195; telephone: 206-543-0724; fax: 206-543-3100; e-mail: candan@u.washington.edu

[2]Molecular Biology and Genetics, Istanbul Technical University, Istanbul 34469, Turkey; telephone: +90-212-2857252; fax: +90-212-2856386; e-mail: tamerler@itu.edu.tr

[3]Department of Chemistry, University of Washington, Seattle, Washington 98195

[4]Department of Chemical Engineering, University of Washington, Seattle, Washington 98195

Received 27 October 2008; revision received 26 January 2009; accepted 2 February 2009

Published online 9 February 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bit.22282

ABSTRACT: Current biotechnological applications such as biosensors, protein arrays, and microchips require oriented immobilization of enzymes. The characteristics of recognition, self-assembly and ease of genetic manipulation make inorganic binding peptides an ideal molecular tool for site-specific enzyme immobilization. Herein, we demonstrate the utilization of gold binding peptide (GBP1) as a molecular linker genetically fused to alkaline phosphatase (AP) and immobilized on gold substrate. Multiple tandem repeats ($n = 5, 6, 7, 9$) of gold binding peptide were fused to N-terminus of AP (nGBP1-AP) and the enzymes were expressed in *E. coli* cells. The binding and enzymatic activities of the bi-functional fusion constructs were analyzed using quartz crystal microbalance spectroscopy and biochemical assays. Among the multiple-repeat constructs, 5GBP1-AP displayed the best bi-functional activity and, therefore, was chosen for self-immobilization studies. Adsorption and assembly properties of the fusion enzyme, 5GBP1-AP, were studied via surface plasmon resonance spectroscopy and atomic force microscopy. We demonstrated self-immobilization of the bi-functional enzyme on micro-patterned substrates where genetically linked 5GBP1-AP displayed higher enzymatic activity per area compared to that of AP. Our results demonstrate the promising use of inorganic binding peptides as site-specific molecular linkers for oriented enzyme immobilization with retained activity. Directed assembly of proteins on solids using genetically fused specific inorganic-binding peptides has a potential utility in a wide range of biosensing and bioconversion processes.

Biotechnol. Bioeng. 2009;103: 696–705.

KEYWORDS: inorganic binding peptides; enzymes; oriented-immobilization; genetic fusion; self-assembly

Correspondence to: Prof. C. Tamerler

Introduction

Immobilization of proteins through self-assembly onto solid surfaces with high affinity and material selectivity is an essential component in building up protein chips, protein microarrays, and biosensors (Bailey et al., 2007; Jung et al., 2008; Kwon et al., 2006; Rusmini et al., 2007; Tomizaki et al., 2005; Watzke et al., 2006). Although, there are many methods for protein immobilization on to solid surfaces, site-specific protein immobilization has been increasingly used to maintain protein conformation and retained biological activity on selected solid surfaces (Kumada et al., 2006; Kwon et al., 2006; Park et al., 2006). Existing protein immobilization methods are mostly based on non-specific adsorption via, for example, intermolecular forces, mainly ionic bonds and hydrophobic and polar interactions, or chemical coupling reactions which mostly lead to uncontrolled attachment of the proteins on to solid surfaces (Kwon et al., 2006; Rusmini et al., 2007). For example, self-assembled monolayers (SAMs) of thiol- and silane-based molecules on solid substrates (i.e., gold and silica, respectively) are widely used for various chemical and biological surface functionalization (Babu et al., 2004; Neves-Petersen et al., 2006; Mrksich and Whitesides, 1996). These synthetic molecules provide an intermediary molecular layer between the surface and the functional proteins. Using thiol or silane molecules as linkers, proteins are usually immobilized on to solid surfaces in a random orientation, often resulting in a limited or loss of biological activity since the site-specific chemical reaction between functional groups on protein and the activated support surface can still be difficult to control. Moreover, this approach requires complex chemistries for efficient coupling (Norde, 1986; Zhen et al., 2006) which,

often, may not be biocompatible. There are also further limitations in these process in which, for example, in the case of gold surface, alkanethiol layers rapidly degrade due to oxidation (Park et al., 2006; Willey et al., 2005). Current approaches, therefore, are a result of a compromise between maintaining high activity of the enzyme while having the advantage of solid immobilization via self-assembly (Bornscheuer, 2003).

Inorganic-binding peptides may offer advantages compared to synthetic-based molecular systems in the immobilization of functional proteins on to solid surfaces because of their large variety of biochemical and molecular characteristics (Fairman and Akerfeldt, 2005; Krauland et al., 2007; Sarikaya et al., 2003). During the last decade, combinatorial biology libraries have been employed for selection of inorganic binding peptides which are utilized as biosynthesizers, molecular linkers, and assemblers (Brown et al., 2000; Kramer et al., 2004; Kriplani and Kay, 2005; Sano et al., 2005; Sarikaya et al., 2003; Whaley et al., 2000). Furthermore, solid-binding peptides were also selected for synthetic polymeric materials (Adey et al., 1995; Kenan et al., 2006). Molecular biology protocols further allow tailoring of the selected peptides to tune their binding and material-selective properties so that they can be used in bionanotechnological applications, such as linkers for solid colloidal nanoparticles and flat substrates (e.g., gold or glass) (Kacar et al., 2009; Tamerler et al., 2006a). Recently they are also used as part of a fusion protein in the development of multifunctional molecular constructs for a variety of practical applications (Dai et al., 2005; Holmes, 2002; Johnson et al., 2008; Kramer et al., 2004; Sanchez et al., 2005). Such hybrid platforms, containing genetically fused proteins with site-specific solid-binding peptides, could be potential novel ways of efficient immobilization of enzymes and receptors under ambient conditions with wide variety of applications (Brown, 1997; Ishikawa et al., 2008; Krauland et al., 2007; Kumada et al., 2006; Park et al., 2006; Zhang and Cass, 2001).

Gold binding peptides, selected by cell surface display (Brown, 1997), are one of the first examples of engineered peptides for inorganic surfaces (Sarikaya et al., 2003). They were screened via random peptide libraries expressed on the outer surface of *E. coli* as part of the maltodextrin porin, LamB protein. Through a biopanning process, the surface bound bacteria were selected and the displayed peptide sequences were identified. Among the selected peptides, GBP1 (MHGKTQATSGTIQS) has been well characterized by our collaborative group (Brown et al., 2000; Tamerler et al., 2006a,b). This motif does not contain cysteine which is known to form a covalent thiol linkage to gold, as in thiolated molecules that form SAMs. Naturally occurring inorganic binding proteins commonly posses repeating peptide domains, such as ice binding protein (Sicheri and Yang, 1995), silaffin (Kroger et al., 2001), and collagen (Rich and Crick, 1955), providing a robust architecture and ability to self-assemble. Inspired by biology, we designed multiple repeats of gold binding peptide and demonstrated that tandem repeats, in particular the 3-repeat GBP1, enhances the gold binding activity (Tamerler et al., 2006b). We also demonstrated that micro-patterned 3GBP1 can act as a molecular template for direct assembly of gold nanoparticles (Zin et al., 2005). The GBP1 has proven to be an effective linker for the gold surface as it is evidenced from the increased interest and utility in the literature.

Recently, immobilization of enhanced green fluorescent protein, severe acute respiratory syndrome (SARS) coronavirus envelope protein and core streptavidin of *Streptomyces avidinii* on gold surface was achieved in conjunction with GBP1-6Histidine as fusion partner (Park et al., 2006). However, histidine-tags are known to bind to gold substrate (Peelle et al., 2005; Presnova et al., 2000; Slocik and Wright, 2003). The presence of histidine-tag, therefore, precludes the effect of GBP1 as a molecular linker for immobilization of proteins on to gold. In an earlier study, using a GBP construct, a surface plasmon resonance (SPR) biosensor was also constructed (Woodbury et al., 1998). Here, the seven-repeat gold binding peptide (7GBP1-AP) was assembled on SPR substrate and then AP was cleaved off by trypsin from an unspecified region of GBP1, possibly still leaving a residual bound GBP1 layer on to gold which was then used to detect antibodies. Although these studies attempted to use GBP as a molecular linker, the full potential of GBP1 has not been demonstrated. First of all, producing peptide-based surface functionalization requires an examination of solid-adsorption and self-assembly properties with the isolated, full length of the peptide displayed (Seker et al., 2007; Tamerler et al., 2006b). The utility of inorganic-binding peptides, such as GBP1, as part of a fusion protein, as we demonstrate here, is a potential approach for effectively immobilizing proteins on gold surface with retained activity.

Alkaline phosphatase (AP, EC 3.1.3.1) is a dimer hydrolase, widely used in immunoassays for removing inorganic phosphate groups from various types of molecules such as proteins and DNA (Anderson et al., 1975). The wide utility of AP in biochemical, immunological, and medical assays resulted in numerous studies investigating the immobilization of AP via fusion peptides on solid supports. In previous studies, binding of recombinant AP to various surfaces was shown via non-specific peptide tags (Tominaga et al., 2005; Zhang and Cass, 2001) and epitope conjugates of antibodies (Brennan et al., 1995). Here, we demonstrate the utility of an inorganic binding peptide as molecular linker genetically fused to bacterial AP for site-specific immobilization on the metal surface.

We used both AP and GBP1-AP fusion proteins which were expressed in *E. coli* cultures and then purified. Several tandem repeats of the GBP1 were tested to assess changes in gold binding and enzymatic activities of the fusion protein. The bi-functional activities were characterized systematically using various spectroscopic and imaging methods. Assembly characteristics of the fusion proteins were studied by atomic force microscopy (AFM) while their adsorption on to gold was quantified using quartz crystal microbalance

(QCM) and SPR spectroscopy. The oriented immobilization of recombinant AP with the highest enzymatic activity was optimized via the site-specific linkage of the five-repeat gold binding peptide. Furthermore, we also demonstrated the effectiveness of the directed self-assembly of the recombinant bi-functional enzyme on to patterned gold regions prepared by micro-contact printing.

Materials and Methods

Chemicals

Isopropylthiogalactopyranoside (IPTG), *p*-nitrophenyl phosphate (*p*NPP) and diethylaminoethyl (DEAE) Sephacel were purchased from Sigma-Aldrich (St. Louis, MO). Amicon Ultra centrifugal filter devices (10 kDa cut off) were from Millipore (Billerica, MA), Sephacryl HS200 was from Amersham Biosciences (Piscataway, NJ). Finally, Coomassie Plus Protein Assay Reagent used was supplied by Pierce Company (Rockford, IL).

Strain and Plasmids

The *E. coli* S2157 cells harboring either the plasmid pSB2991 that encodes AP or one of the plasmids, i.e., pSB3057, pSB3055, pSB3053, and pSB3127 encoding 5-, 6-, 7-, and 9-repeat tandem gold binding polypeptide fused to AP, respectively, were provided by S. Brown (University of Copenhagen, Denmark).

Growth and Purification of Enzymes

Each cell culture of S2157 with one of the plasmids was grown in yeast extract tripton (YT) medium containing 100 μg/mL ampicillin at 34°C with shaking until the absorbance at 600 nm of 0.5. Expression was induced by 1 mM of IPTG for 6 h. Subsequently, the cells were harvested from the broth by centrifugation. Next, the periplasmic fraction containing the enzyme was isolated by cold osmotic shock protocol. Prior to purification, all buffers were prepared freshly and 1 mM phenyl methane sulphony fluoride (PMSF) was added as protease inhibitor. The shock fluid was concentrated by Amicon Ultra centrifugal filter device and passed through DEAE-Sephacel column equilibrated in 20 mM Tris–HCl (pH 8.0) buffer. After washing with the same buffer, enzyme fractions were eluted with 20 mM Tris–HCl, making a gradient from 0.0 to 0.1 M NaCl. The fractions containing the desired protein were pooled and concentrated to 5.0 mL by 10 kDa Millipore and passed over Sephacryl HS200 column in 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1.0 mM $MgCl_2$. The eluted fractions containing the purified enzyme were then pooled. Protein concentration was determined by using Bradford protein assay with bovine serum albumin as the standard. Protein fractions at each step of purification were analyzed by SDS/PAGE (10% (w/v) gel) under denaturing conditions and the proteins were stained with Coomassie Blue (Bradford, 1976; Brown et al., 2000).

Preparation of Micro-patterned Gold Substrates

The silicon wafers coated with gold were prepared by electron-beam evaporation of gold (~25 nm thick) onto single-crystal silicon (100) oriented test wafers (Silicon Sense, Nashua, NH; 100 mm in diameter, ~500 μm thick) that had been pre-coated with a layer of titanium (~2 nm thick). The substrates were fractured into slides (~2 cm × 2 cm) for micro-contact printing (μCP) of oligo (ethylene glycol)-terminated alkanethiols (OH-$(OCH_2)_3$-SH). Photoresist micro-patterned masters, which contained a negative relief of the stamp, were fabricated at the Microfabrication Lab (Washington Technology Center) at the University of Washington. The stamps were made by casting a 10:1 (v/v) mixture of polydimethyl siloxane and curing agent (Sylgard 184, Dow Corning, Midland, MI) against a silanized master for 2 days at ambient conditions. Freshly prepared stamps were washed several times with ethanol, heptane and, lastly, with ethanol and then dried in a flow of nitrogen before being used. The inking was performed by rubbing a Q-tip soaked with the ethanolic solution of OH-$(OCH_2)_3$-SH (5 mM) across the surface of the stamp. The inked stamp was dried in nitrogen and brought into a conformal contact with the gold surface for ~20 s. The patterned substrates were rinsed copiously in ethanol, dried under nitrogen, and were used immediately for the self-assembly of the proteins. After each cycle of inking and printing, the stamps were cleaned by ultrasonication in a 2:1 solution of water and ethanol for 5 min.

Enzyme Activity Assay

Various concentrations (0.5–3.0 μg/mL) of enzyme in 10 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ (Reaction buffer) were assayed with 5.5 mM *p*NPP for 30 min. The release of *p*-nitrophenol (*p*NP) was recorded by 96-well TECAN plate reader (San Jose, CA) at 405 nm, 37°C. The AP activity was calculated from Beer-Lambert law as follows (Eq. 1):

$$\text{Enzymatic activity (mmol/min)} = \frac{V(\text{ml}) \times \text{OD}_{405\text{nm}}(\text{cm}^{-1})}{e \times \text{incubation time (min)}} \quad (1)$$

where ε is the molar extinction coefficient (M^{-1} cm^{-1}) which for *p*NP is $\varepsilon = 1.78 \times 10^4$ M^{-1} cm^{-1}; V is the final assay volume; OD_{405nm} (cm^{-1}) is the absorbance divided by the light-path length (cm). After calculating the average of the triplicate assays, the blank values were subtracted from those of OD_{405nm}.

Quantification of Immobilized Enzyme Activity

For quantification of enzymatic activity, the reaction buffer (800 μL), containing 3.0 μg/mL of enzyme, was incubated with either non-patterned or micro-patterned gold substrate in microfuge tubes and mixed at room temperature for 16 h. At the end of the incubation period, the substrate was taken out of the tubes and, washed with buffer and DI water solution. These were then dried by a flow of nitrogen before AFM characterization or activity assays (see the conditions in Materials and Methods Section). Also, as a control, the retained activity of the immobilized enzymes was determined by assaying the activity of the free enzyme in solution. The percentage of the retained enzymatic activity on the non-patterned gold surfaces was calculated as follows: the activity on the substrate was divided by that gained from reference solution, that is (Enzyme $\text{Activity}_{5\text{GBP1-AP}}$ on substrate)/(Enzyme $\text{Activity}_{5\text{GBP1-AP}}$ in solution), multiplied by 100.

AFM Measurements

The visualization of the samples was carried out by an AFM using a Digital Instruments Multimode scanning probe microscope with a Nanoscope IIIa controller (Santa Barbara, CA) under acoustic and mechanical isolation. High frequency (~300 kHz) silicon nitride cantilevers were purchased from Molecular Imaging (Tempe, AZ) and used at a scanning velocity of ~3 μm/s for minimal feedback artifacts. Topographic feature sizes and cross-sectional analysis were performed using the Nanoscope software (ver 5.3r1) provided with the AFM by Digital Instruments Co (Santa Barbara, CA).

QCM Experiments

The AT-cut QCM electrodes (zero frequency dependence on temperature), with a fundamental resonant frequency of 10 MHz, were obtained from International Crystal Manufacturing Co. (Oklahoma City, OK). The crystals were coated on both sides with a 100 Å-thick chromium followed by a 1,000 Å-thick gold films. The crystal surfaces were optically polished before metal coating. The diameter of the crystals and electrodes used were 5 and 8.8 mm, respectively. The oscillation electronic circuit was a typical Collpits oscillator, which has a buffer amplifier. The voltage of 12 V DC was applied to the oscillator circuit to drive the crystal and the frequency was measured with a Hewlett-Packard frequency counter (Model No: 53131A 225 Hz Universal Counter, Agilent Technologies, Santa Clara, CA). Before starting the experiments, the crystals with the gold electrode were first cleaned with 1:3 (v/v) 30% H_2O_2/H_2SO_4, "piranha solution," for 5 min at room temperature and then rinsed with DI water. The crystals were then used immediately after they were dried under a flow of nitrogen gas. To establish a stable baseline, a sufficient volume of the reaction buffer was introduced into the cell before adding the enzyme solution. The frequency change of the crystal in pure buffer was recorded for 30–60 min. Following the equilibration with buffer, desired amount of enzyme in buffer was introduced into the cell and the frequency change was recorded continuously.

SPR Experiments

The SPR measurements were carried out with a dual channel instrument (Kretschmann configuration) developed by the *Radio Engineering Institute*, Czech Republic (Seker et al., 2007). This is a generic instrument consisting of a polychromatic light source (Ocean Optics LS1) coupled with an optical fiber and an Ocean Optics SD 2000 detector (Dunedin, FL). The data were collected using WinSpectral 1.03; this software was supplied along with the instrument. This program normalizes the acquired SPR spectrum using the dip in the wavelength shift at regular intervals which then is fit with a 4th-order polynomial for generating the metric sensogram as a function of time. During the experiments, a baseline was established by pumping the reaction buffer first, and then the 6-port valve (Upchurch Scientific, Oak Harbor, WA) was switched to enzyme solution until a full saturation was achieved. The buffer was then pumped once again (at a rate of 80 μL/mL) to monitor the desorption behavior.

The Langmuir isotherm model was used to deduce the kinetics of the adsorption process using the experimental SPR data. Here, we first calculated $k_{\text{observable}}$ using $k_{\text{obs}} = k_a C + k_d$; where C is the enzyme concentration, and k_a and k_d are the association and dissociation constants, respectively. Next, we calculated the equilibrium constant (K_{eq}) and the equilibrium surface coverage (θ) data, using $K_{\text{eq}} = k_a/k_d$ and $\theta = C/(C + K_{\text{eq}}^{-1})$, respectively (Goren et al., 2006; Tamerler et al., 2006b).

Results and Discussion

Optimization of Tandem Multiple Repeat Gold Binding Peptide-Alkaline Phosphatase Fusion Construct

Based on the hypothesis that the increase in the number of the tandem GBP1 repeat would increase the binding activity to gold, in our previous studies, we used 3-repeat GBP1 and showed that, in fact, the binding of 3GBP1 is better than that of single-repeat GBP1 (Tamerler et al., 2006a,b). Starting with a minimum 3GBP1 as a fusion partner to Laccase, we also observed that 5-repeat GBP1 provided a better gold binding activity of this fusion protein (unpublished data). In this work, therefore, we started with 5-repeat GBP1 and increased the number in the tandem sequence ($n = 5, 6, 7, 9$) to evaluate the effect on the bi-functional activities. Multiple tandem repeats of cell surface display-selected gold binding peptide were genetically fused to the enzyme, AP,

at the N-terminus. The plasmids encoding the wild-type AP and the bi-functional constructs (nGBP1-AP) were expressed in *E. coli* S2157 cells. The expressed enzymes were secreted from the cytoplasm to the periplasmic space. Wild-type or bi-functional AP in the periplasmic fluid was purified by employing two successive steps: ion-exchange and gel filtration chromatography. The purified samples were run onto SDS–PAGE to verify their purity and molecular weights (Fig. 1a and b). The protein bands of AP, 5GBP1-AP, 6GBP1-AP, 7GBP1-AP, and 9GBP1-AP were, approximately, at the following kDa values (compared to the theoretical molecular weights in parentheses), respectively: 46.5 (50.1), 54.5 (57.4), 55.3 (58.8), 56.0 (60.3), and 58.0 (63.1). The molecular weights of the proteins purified are comparable to *E. coli* AP, a homodimer with a molecular weight of 89 kDa (Anderson et al., 1975).

The bi-functionality of enzymes was evaluated for both phosphatase and gold binding activities using biochemical, spectroscopic, and molecular imaging protocols. The AP activities of the wild-type protein and fusion constructs were measured spectrophotometrically using *p*NPP as a substrate (Fig. 2a). We then monitored the gold binding activities of bi-functional enzymes on gold electrode using QCM (Fig. 2b). The 5GBP1-AP and 6GBP1-AP presented higher gold binding activity compared to either 7GBP1-AP or 9GBP1-AP. The 5GBP1-AP had also the highest phosphatase activity; this fusion construct, therefore, was chosen to carry out the subsequent directed immobilization studies.

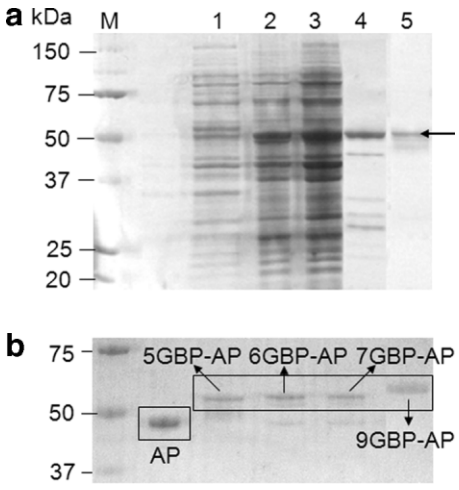

Figure 1. **a**: SDS–PAGE of 5GBP1-AP purification steps. The arrow indicates 54.5 kDa band of 5GBP1-AP. Lane M: Molecular weight marker (Bio-Rad Labs, Hercules, CA) with corresponding molecular masses at the left, **lane 1**: Induced culture, **lane 2**: periplasmic fluid, **lane 3**: periplasmic fraction concentrated by centrifugal filter tube, **lane 4**: DEAE chromatography, **lane 5**: gel filtration chromatography. **b**: The image of 10% SDS gel of the purified constructs with the same molecular weight marker. The gel images were analyzed by Total Lab Software (ver 2.01) to determine the experimental molecular weight of enzymes.

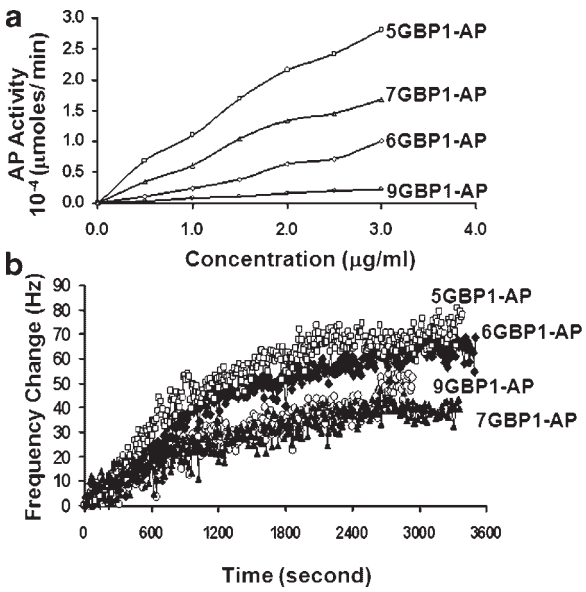

Figure 2. Bi-functional activities of the molecular constructs: (**a**) alkaline phosphatase activity of nGBP1-AP and (**b**) gold binding activity of nGBP1-AP constructs by quartz crystal microbalance analysis (protein concentration: 2.5 μg/mL).

Binding and Assembly Characterization of Bi-Functional 5GBP1-AP on Gold Substrate

The directed immobilization and the resulting morphology of the AP and the 5GBP1-AP on gold substrate were examined using non-contact mode AFM (Fig. 3a–d). The dimensions of the immobilized enzymes obtained from AFM characterization are comparable with the molecular dimensions of the bacterial AP obtained from the Protein Data Bank, as: 9.77 nm × 5.40 nm × 4.75 nm (Protein Data Bank website, http://www.rcsb.org/pdb/home/home. do, 2009). Upon closer inspection in Figure 3, it can be recognized that the 5GBP1-AP units are significantly more discrete and well packed on the surface (Fig. 3a and b) than the wild-type AP (Fig. 3c and d). The peak-to-peak analyses of the topography of the images from the two substrates also yielded insights into the packing density of the particles. In the case of 5GBP1-AP, a higher packing density, with a peak-to-peak separation of 11.0 ± 2.9 nm, was observed compared to AP alone, with $\sim 16.0 \pm 1.5$ nm, as shown in the representative cross-sectional measurements in Figure 3b and d. Since the same tip was used to produce the AFM images from both of the samples, the discrepancy in feature sizes was not seen to arise from experimental artifacts, such as tip convolution. The evidence of agglomeration in the AP sample, without the fusion peptide, can also be recognized by comparing the *z* (height) data between the two sets of images. From Figure 3b and d, the cross sections reveal an average height variation of almost double that of the actual size of the 5GBP1-AP (8 ± 4 nm). This observation suggests that there may be multiple layers of AP in the absence of GBP1, possibly due to agglomeration

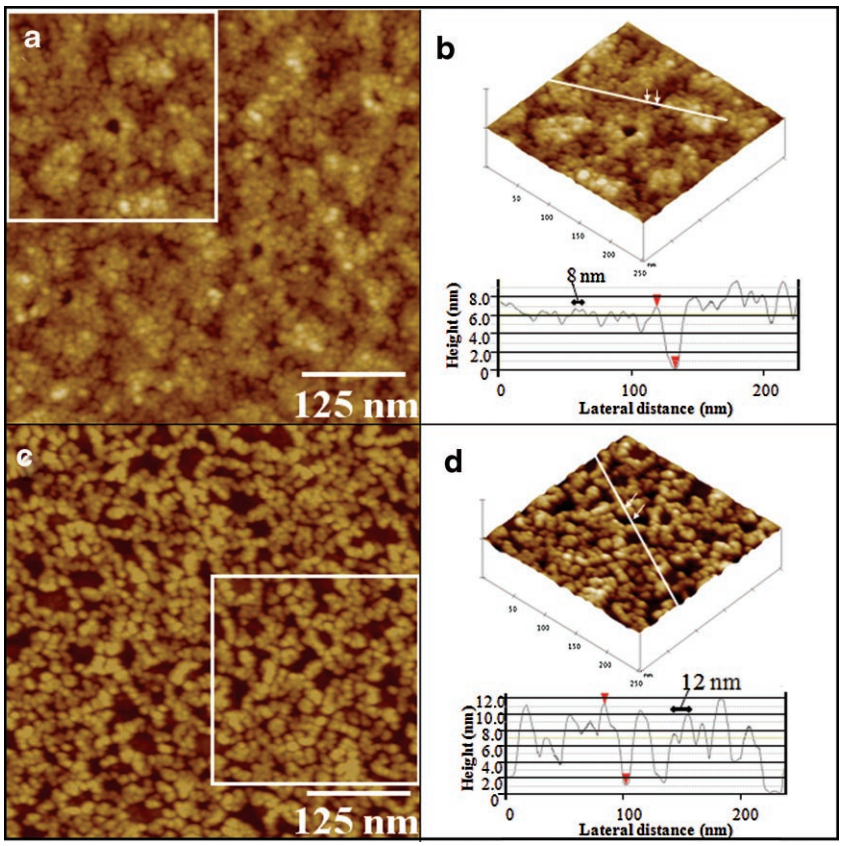

Figure 3. The AFM images demonstrate surface topography of the 5GBP1-AP construct (**a** and **b**) and AP alone (**c** and **d**) at 3 μg/mL. The area corresponds to 500 nm × 500 nm scans. The images in (a and c) are magnified digitally and represented in pseudo-3-dimensional presentation (b and d) at 150 nm × 150 nm area to show surface topography following the immobilization following in the two cases. Discrete enzyme molecules are observed in a and b while the molecular distinction is lost and clusters appear in c and d. The dimensions 8 and 12 nm in b and d are representatives of the peak-to-peak distances shown by double diamonds in the respective AFM images from 5GBP1-AP and the control AP. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

normal to the surface or misorientation of the protein with its longest axis normal to the surface. In the case of the 5GBP-AP construct, a discrete and closely packed monolayer was formed with a thickness closest to the c-dimension of AP, that is, 6 ± 1 nm. This result suggests, therefore, that the enzyme has likely been oriented right side up (i.e., enzymatic sites facing the solution) by the gold binding peptide. Additionally, Figure 3a and c also show undulating plateau-like features throughout the surface of about ~30 nm, indicative of the polycrystalline gold grain structure of the underlying surface. With these observations and peak-to-peak measurements, it can be reasoned that the surface in Figure 3a and b is more indicative of a uniformly thin and oriented molecular layer than those observed in Figure 3c and d where AP was used.

The SPR experiments were also conducted on gold substrate to study binding kinetics of the fusion product. Through these studies, we also calculated the overall coverage of the enzyme on the gold surface. The control experiments for binding studies were first carried out using the control AP where we know that the enzyme alone can non-specifically bind to gold. The molecular adsorption profiles of the 5GBP1-AP, however, reveal a significant increase in adsorption, due to material specific binding activity provided to the enzyme by the gold binding peptide (Fig. 4a). As a result, based on the Langmuir adsorption model fit to both sets of the SPR data, we find that 5GBP1-AP displays a higher surface coverage than that of the control AP (Fig. 4b). Based on the model, the calculated kinetic parameters for both enzymes are tabulated in Table I. Depending on the concentration used, 5GBP1-AP reached to nearly 90% surface coverage with an equilibrium adsorption constant (K_{eq}) of 1.65×10^8. This value is comparable to the adsorption of alkanethiol on gold, the first step for the gold surface functionalization prior to protein immobilization in conventional chemical approaches (Karpovich and Blanchard, 1994; Schessler et al., 1996).

Peptide Mediated Self-Immobilization of Enzyme on Micro-Patterned Surfaces

Protein array technologies necessitate efficient patterning of biomolecules on selected micro-patterned substrates providing spatial immobilization on an inorganic surface.

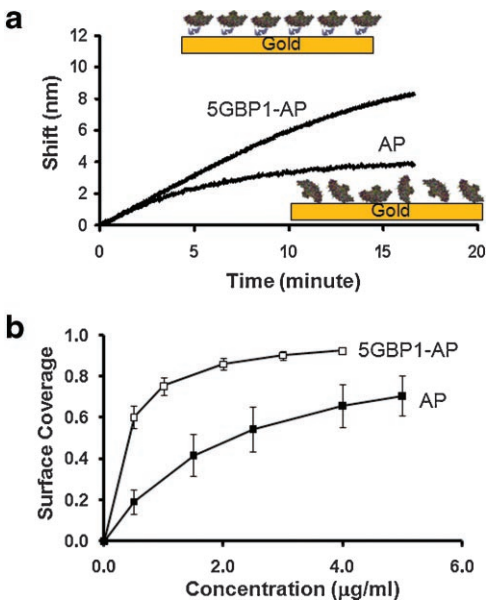

Figure 4. **a**: SPR spectroscopy results of AP and 5GBP1-AP binding to bare gold surfaces at 4 μg/mL concentration ("Shift" corresponds to the change in the dip position of the SPR spectrum). **b**: Surface coverage of AP and 5GBP1-AP has been calculated using the Langmuir isotherm model based on the SPR experiments. The schematics in (a) depict possible scenarios for directed and non-specific immobilization of the enzyme, respectively. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

This is possible using various lithography techniques, for example, soft lithography (Xia and Whitesides, 1998), dip-pen lithography (Lee et al., 2002), and photolithography (Revzin et al., 2001). Taking advantage of the gold binding activity of the 5GBP1-AP fusion construct, in this part of our work, we studied whether the enzyme could be directed-assembled on a patterned substrate. Among a variety of pattern fabrication methods, micro-contact printing (μCP) has proven to be a versatile technique which does not necessitate the use of expensive traditional lithographic equipments (Xia and Whitesides, 1998). Using μCP, therefore, a variety of biological molecules can be patterned on solid surfaces with sub-micron features over a large area (>1 cm^2). For pattern formation, oligo (ethylene glycol)-terminated alkanethiols (OH-$(OCH_2)_3$-SH) are generally used which are known to resist protein adsorption on to gold (Prime and Whitesides, 1993). The remaining, bare, gold regions are then functionalized using thiolate molecules designed for the desired protein coupling. Rather than the two-stage thiol linkage for protein immobilization, in our work, we examined the peptide-based linkage of the fusion enzyme. It should be pointed out that the position of the GBP insertion, at the N-terminus of AP, is opposite to the active site of the enzyme (as depicted in the inset of Fig. 4a).

As demonstrated in Figure 5, and described in the Materials and Methods Section, we prepared micro-patterned substrates that effectively cover large areas of the surface (millimeters) with a SAM, specifically, oligo (ethylene glycol)-terminated alkanethiols, providing a few μm-diameter circular, bare gold regions. Using either the AP alone or the 5GBP1-AP fusion, we studied to see if there was any effect of the immobilization protocol on the efficiency of adsorption and in the enzyme activity. The AFM images (Fig. 5b–e) show that both the wild-type and the hybrid construct bind to the gold regions of the patterned surface. However, the molecular packing is denser (high number density of enzyme immobilized) and more homogenous in the case of 5GBP1-AP (Fig. 5b vs. d) compared to the wild-type AP. In fact, the coverage area of

Table I. The parameters of binding kinetics for 5GBP1-AP and AP on bare Au surfaces obtained by SPR.

	k_a [$M^{-1}s^{-1}$]	k_d [s^{-1}]	K_{eq} [M^{-1}]
AP	$1.35 \pm 0.26 \times 10^4$	$6.00 \pm 0.16 \times 10^{-4}$	$2.24 \pm 0.22 \times 10^7$
5GBP1-AP	$3.19 \pm 0.12 \times 10^4$	$1.94 \pm 0.05 \times 10^{-4}$	$1.65 \pm 0.12 \times 10^8$

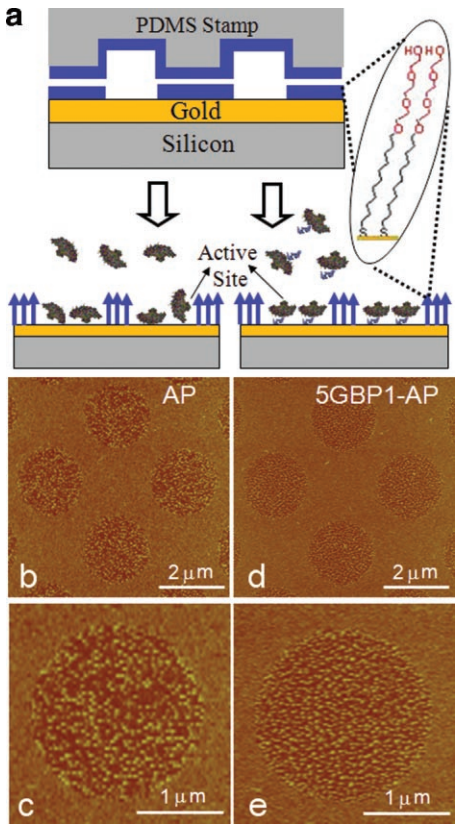

Figure 5. **a**: Schematic representation of the experimental procedure for the generation of two-dimensional arrays of immobilized proteins on a patterned substrate fabricated through micro-contact printing (black arrows show active site of the enzyme). Topographic images of the micro-arrays of immobilized AP (**b** and **c**) and 5GBP1-AP (**d** and **e**) were recorded by an AFM in tapping mode (silicon cantilever, scan rate = 1.5 Hz, spring constant 40 N/m) at different magnifications. [Color figure can be seen in the online version of this article, available at www.interscience.wiley.com.]

5GBP1-AP, determined by AFM, was ~40% higher than that of AP alone (Fig. 5c vs. e).

In the present experiments, the enzyme was immobilized on the gold substrate while keeping the same amount of free enzyme in solution as the reference. The prepared solution, incubated at room temperature, was assayed to quantify the enzymatic activity (see Materials and Methods Section) which was assessed under several different conditions. First, we estimated how much of the free enzyme in solution was transferred to the non-patterned gold surface. To assess this, we calculated the percentage of retained enzymatic activity by taking the ratio of the activity on the surface to that in the solution. We found that, when immobilized, only 2% (±0.4) of wild-type AP activity was transferable onto non-patterned gold surface, whereas in case of 5GBP1-AP, transferable activity to the surface was 66% (±0.6). The enzymatic activities obtained from both the non-patterned and the micro-patterned substrates were normalized by the corresponding surface area of the substrate available for binding (Fig. 6). This yielded a value (in $\mu mol/min/\mu m^2$) of ~1.8 times higher for 5GBP1-AP as compared to the wild-type AP even on the non-patterned surfaces. This result also supports our earlier findings discussed above in reference to the AFM (Fig. 3) and SPR (Fig. 4) analyses in which higher and more homogenous coverage of 5GBP1-AP were observed compared to a lower coverage and non-homogenous immobilization of the wild-type AP. Similar to above, we also investigated the enzyme activity on the micro-patterned-substrates (Fig. 5). In general, one observes an increase in the enzymatic activity of both the wild-type and fusion enzymes when targeted immobilized on the micro-patterned surfaces (Fig. 6). The increase, however, was more prominent in the fusion protein with the GBP linker than when this linker was absent. In fact, the immobilization of the AP enzyme with GBP linker on the patterned surface resulted in three times higher enzymatic activity compared to the wild-type AP on the non-patterned substrate. Here, the double-advantage of using genetically engineered peptide for inorganics (GEPI) as the fusion linker as well as the effect of assembly in a confined area as a result of patterning was effective simultaneously to result in enhanced performance of the enzyme. While a GEPI allows the control of immobilization of the enzyme relative to the substrate, the high number density of packing possibly increases the folding stability of the protein (Zhou and Dill, 2001).

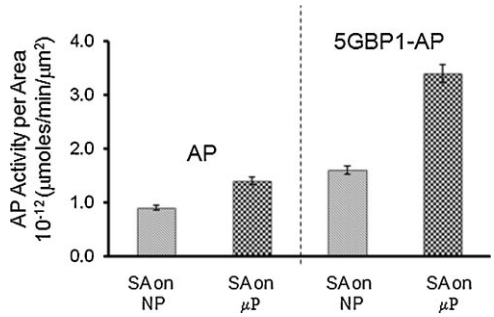

Figure 6. The calculated AP activities per unit area for AP and 5GBP1-AP corresponding to self-assembly (SA) of each enzyme on the non-patterned (NP) and micro-patterned (μP) gold substrates. The AP activity per unit area for both enzymes was obtained by normalizing the activity of enzyme by the corresponding surface area available (22.4×10^6 and 64.0×10^6 μm^2 for micro-patterned and non-patterned, respectively).

The key parameter in the process of immobilization of enzymes onto a solid surface is the ability to keep the active site available for the catalytic reactions for high efficiency while maintaining the stability of the enzyme on the solid surface (Kasemo, 2002; Shao et al., 2000). Various residues or domains of a given protein may interact with a given solid. These interactions are often non-specific and result in protein adsorption with loss of function as well as a loss of long-term stability (Zhang and Cass, 2001). As schematically shown in the inset of Figure 4a, non-specific binding of AP may lead to the blocking of the active site of the enzyme which, in turn, may cause a loss of AP activity. In this context, the GBP1 linkage provides an oriented self-immobilization of the enzyme with retained activity, as demonstrated here. Our results also show that enzymatic activity per unit area can be enhanced by directing the fusion construct on to spatial locations on a micro-patterned surface via directed assembly using the inorganic-binding peptide. Here, it may be suggested that directed assembly provides the self-localization of the molecular construct on a confined surface leading to a higher number of protein adsorption per unit surface area. Therefore, the inorganic binding peptide, that is, GBP1, not only provides specific adsorption onto gold substrate but it also, through its genetic fusion, allows oriented immobilization of enzyme leaving its catalytic site available to carry out the reactions relatively freely. The molecular platform used here can be utilized successfully for self-immobilization of enzymes in their biologically active state on any solid materials (silica, graphite, etc.) using the appropriate GEPI linker specific to that substrate, for example, silica binding peptide for silica surface, graphite-binding peptide for graphite, and gold binding peptide for gold surface.

As we observed, when measured in solution, the enzyme activity of the bare AP (28.8×10^{-4} μmol/min) is approximately fifteen times higher than that of the 5GBP1-AP fusion protein. Therefore, it appears that the enzyme does lose its activity in the hybrid form. However, this should not necessarily be a general trend for all enzymes. In the present work, we prepared the hybrid construct by fusing the gold binding peptide on to the N-terminus of the AP which may not be the ideal site for fusion. We think, therefore, that to obtain the optimum enzymatic performance, one may need to perform a genetic search for a permissive site (Manoil and Traxler, 2000). The knowledge of the permissive site, therefore, would provide the position for genetic fusion of the inorganic-binding peptide on to the molecule with the highest retained enzymatic activity while

also allowing solid binding functionality of the fusion. Furthermore, many proteins do bind to gold surface non-specifically; therefore other solids, most notably, silica, may be a better substrate for enzyme immobilization through inorganic silica-binding peptide (Kacar et al., 2009). Finally, the possible ''confinement effect'' observed here on the patterned surface caused higher enzyme activity because of the higher number of ''correctly'' immobilized and homogeneously distributed enzymes in a confined space. This may be due to the limited surface area available for enzyme immobilization for a given concentration from the solution. While the inorganic binding activity of the fused GEPI allows solid binding and proper display (orientation) of the enzyme on the solid surface, the area confinement may also increase the high density assembly and, therefore, the activity of the enzyme. Therefore, it may be possible to observe the same effect when hybrid enzymes are immobilized on nanoparticles of specific size and morphology (limiting surface and controlling orientation). These approaches will undoubtedly be explored by us and, possibly, by others in the near future.

Conclusions

We presented here the utilization of inorganic binding peptides as molecular linkers for the immobilization of enzymes on solid materials. To prove the concept, we used cell surface display-selected gold binding peptide (GBP1) to self-immobilize AP on a gold surface. The enzyme genetically fused to multiple repeats ($n = 5, 6, 7, 9$) of GBP1 were expressed in *E. coli* cells. The bi-functional activity of the construct, that is, both the gold binding and phosphatase activities, was conserved as demonstrated by spectroscopic and biochemical assays. The hybrid enzyme construct that displayed the highest bi-functional activity was selected for self-immobilization experiments. Both spectroscopic and imaging assays showed that gold-specific linkage provided by GBP1 resulted in higher enzymatic activity compared to the wild-type AP. GBP1 mediated AP immobilization, therefore, provided easier access of the enzyme's active site to the surrounding aqueous media. We also showed that μCP fabricated micro-patterned substrates can be used to increase the number density of the self-immobilized enzymes by providing targeted assembly through a guidance during the assembly process. The consequence of the targeted immobilization of the genetically linked GBP1 is a simultaneous effect of directed self-assembly and higher AP activity per area compared to the wild-type. The new directed immobilization process is simple and takes place in aqueous environment requiring no synthetic linkers or special solutions. Self-assembly of the inorganic binding peptide-linked enzyme on the surface is completed fairly rapidly, that is, within hours. Our method is universal and could be extendable to any solid surface such as platinum, graphite, and silica with the use of appropriate inorganic-binding peptides. Although we show here the workings of a gold substrate, the method may be applicable in multi-material patterned functional platforms (Tamerler et al., 2006a) that are addressed through specific inorganic-binding peptide tags, efficient and utilizable for a wide range of applications in bionanotechnology.

This work was supported by grants from National Science Foundation through the NSF-MRSEC Program at Genetically Engineered Materials Science & Engineering Center (GEMSEC) and Turkish State Planning Organization and TUBITAK/NSF-IRES joint project 107T250 (NGK, TK and CT, partially).

References

Adey NB, Mataragnon AH, Rider JE, Carter JM, Kay BK. 1995. Characterization of phage that bind plastic from phage-displayed random peptide libraries. Gene 156(1):27–31.

Anderson RA, Bosron WF, Kennedy FS, Vallee BL. 1975. Role of magnesium in Escherichia-coli alkaline-phosphatase. Proc Natl Acad Sci USA 72(8):2989–2993.

Babu VRS, Kumar MA, Karanth NG, Thakur MS. 2004. Stabilization of immobilized glucose oxidase against thermal inactivation by silanization for biosensor applications. Biosens Bioelectron 19(10):1337–1341.

Bailey RC, Kwong GA, Radu CG, Witte ON, Heath JR. 2007. DNA-encoded antibody libraries: A unified platform for multiplexed cell sorting and detection of genes and proteins. J Am Chem Soc 129(7):1959–1967.

Bornscheuer UT. 2003. Immobilizing enzymes: How to create more suitable biocatalysts. Angew Chem Int Ed 42(29):3336–3337.

Bradford MM. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem 72(1–2):248–254.

Brennan CA, Christianson K, Lafleur MA, Mandecki W. 1995. A molecular sensor system based on genetically-engineered alkaline-phosphatase. Proc Natl Acad Sci USA 92(13):5783–5787.

Brown S. 1997. Metal-recognition by repeating polypeptides. Nat Biotechnol 15(3):269–272.

Brown S, Sarikaya M, Johnson E. 2000. A genetic analysis of crystal growth. J Mol Biol 299(3):725–735.

Dai HX, Choe WS, Thai CK, Sarikaya M, Traxler BA, Baneyx F, Schwartz DT. 2005. Nonequilibrium synthesis and assembly of hybrid inorganic-protein nanostructures using an engineered DNA binding protein. J Am Chem Soc 127(44):15637–15643.

Fairman R, Akerfeldt KS. 2005. Peptides as novel smart materials. Curr Opin Struct Biol 15(4):453–463.

Goren M, Galley N, Lennox RB. 2006. Adsorption of alkylthiol-capped gold nanoparticles onto alkylthiol self-assembled monolayers: An SPR study. Langmuir 22(3):1048–1054.

Holmes TC. 2002. Novel peptide-based biomaterial scaffolds for tissue engineering. Trends Biotechnol 20(1):16–21.

Ishikawa K, Yamada K, Kumagai S, Sano KI, Shiba K, Yamashita I, Kobayashi M. 2008. Adsorption properties of a gold-binding peptide assessed by its attachment to a recombinant apoferritin molecule. Appl Phys Expr 1(3):034006-1–034006–3.

Johnson S, Evans D, Laurenson S, Paul D, Davies AG, Ferrigno PK, Walti C. 2008. Surface-immobilized peptide aptamers as probe molecules for protein detection. Anal Chem 80(4):978–983.

Jung YW, Kang HJ, Lee JM, Jung SO, Yun WS, Chung SJ, Chung BH. 2008. Controlled antibody immobilization onto immunoanalytical platforms by synthetic peptide. Anal Biochem 374(1):99–105.

Kacar T, Ray J, Gungormus M, Oren EE, Tamerler C, Sarikaya M. 2009. Quartz binding peptides as molecular linkers towards fabricating multifunctional micropatterned substrates. Adv Mater 21:295–299.

Karpovich DS, Blanchard GJ. 1994. Direct measurement of the adsorption-kinetics of alkanethiolate self-assembled monolayers on a microcrystalline gold surface. Langmuir 10(9):3315–3322.

Kasemo B. 2002. Biological surface science. Surf Sci 500(1–3):656–677.
Kenan DJ, Walsh EB, Meyers SR, O'Toole GA, Carruthers EG, Lee WK, Zauscher S, Prata CAH, Grinstaff MW. 2006. Peptide-PEG amphiphiles as cytophobic coatings for mammalian and bacterial cells. Chem Biol 13(7):695–700.
Kramer RM, Li C, Carter DC, Stone MO, Naik RR. 2004. Engineered protein cages for nanomaterial synthesis. J Am Chem Soc 126(41): 13282–13286.
Krauland EM, Peelle BR, Wittrup KD, Belcher AM. 2007. Peptide tags for enhanced cellular and protein adhesion to single-crystal line sapphire. Biotechnol Bioeng 97(5):1009–1020.
Kriplani U, Kay BK. 2005. Selecting peptides for use in nanoscale materials using phagedisplayed combinatorial peptide libraries. Curr Opin Biotechnol 16(4):470–475.
Kroger N, Deutzmann R, Sumper M. 2001. Silica-precipitating peptides from diatoms—The chemical structure of silaffin-1A from Cylindrotheca fusiformis. J Biol Chem 276(28):26066–26070.
Kumada Y, Tokunaga Y, Imanaka H, Imamura K, Sakiyama T, Katoh S, Nakanishi K. 2006. Screening and characterization of affinity peptide tags specific to polystyrene supports for the orientated immobilization of proteins. Biotechnol Prog 22(2):401–405.
Kwon Y, Coleman MA, Camarero JA. 2006. Selective immobilization of proteins onto solid supports through split-intein-mediated protein trans-splicing. Angew Chem Int Ed 45(11):1726–1729.
Lee KB, Park SJ, Mirkin CA, Smith JC, Mrksich M. 2002. Protein nanoarrays generated by dip-pen nanolithography. Science 295(5560):1702–1705.
Manoil C, Traxler B. 2000. Insertion of in-frame sequence tags into proteins using transposons. Methods 20(1):55–61.
Mrksich M, Whitesides GM. 1996. Using self-assembled monolayers to understand the interactions of man-made surfaces with proteins and cells. Annu Rev Biophys Biomol Struct 25:55–78.
Neves-Petersen MT, Snabe T, Klitgaard S, Duroux M, Petersen SB. 2006. Photonic activation of disulfide bridges achieves oriented protein immobilization on biosensor surfaces. Protein Sci 15(2):343–351.
Norde W. 1986. Adsorption of proteins from solution at the solid-liquid interface. Adv Colloid Interface Sci 25(4):267–340.
Park TJ, Lee SY, Lee SJ, Park JP, Yang KS, Lee KB, Ko S, Park JB, Kim T, Kim SK, Shin YB, Chung BH, Ku SJ, Kim DH, Choi IS. 2006. Protein nanopatterns and biosensors using gold binding polypeptide as a fusion partner. Anal Chem 78(20):7197–7205.
Peelle BR, Krauland EM, Wittrup KD, Belcher AM. 2005. Design criteria for engineering inorganic material-specific peptides. Langmuir 21(15): 6929–6933.
Presnova G, Grigorenko V, Egorov A, Ruzgas T, Lindgren A, Gorton L, Borchers T. 2000. Direct heterogeneous electron transfer of recombinant horseradish peroxidases on gold. Faraday Discuss 116(116):281–289.
Prime KL, Whitesides GM. 1993. Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide)—A model system using self-assembled monolayers. J Am Chem Soc 115(23):10714–10721.
Revzin A, Russell RJ, Yadavalli VK, Koh WG, Deister C, Hile DD, Mellott MB, Pishko MV. 2001. Fabrication of poly(ethylene glycol) hydrogel microstructures using photolithography. Langmuir 17(18):5440–5447.
Rich A, Crick FHC. 1955. Structure of collagen. Nature 176(4489):915–916.
Rusmini F, Zhong ZY, Feijen J. 2007. Protein immobilization strategies for protein biochips. Biomacromolecules 8(6):1775–1789.
Sanchez C, Arribart H, Guille MMG. 2005. Biomimetism and bioinspiration as tools for the design of innovative materials and systems. Nat Mater 4(4):277–288.
Sano KI, Sasaki H, Shiba K. 2005. Specificity and biomineralization activities of Ti-binding peptide-1 (TBP-1). Langmuir 21(7):3090–3095.
Sarikaya M, Tamerler C, Jen AKY, Schulten K, Baneyx F. 2003. Molecular biomimetics: Nanotechnology through biology. Nat Mater 2(9):577–585.
Schessler HM, Karpovich DS, Blanchard GJ. 1996. Quantitating the balance between enthalpic and entropic forces in alkanethiol/gold monolayer self assembly. J Am Chem Soc 118(40):9645–9651.
Seker UOS, Wilson B, Dincer S, Kim IW, Oren EE, Evans JS, Tamerler C, Sarikaya M. 2007. Adsorption behavior of linear and cyclic genetically engineered platinum binding peptides. Langmuir 23(15):7895–7900.
Shao WH, Zhang XE, Liu H, Zhang ZP. 2000. Anchor-chain molecular system for orientation control in enzyme immobilization. Bioconjug Chem 11(6):822–826.
Sicheri F, Yang DSC. 1995. Ice-binding structure and mechanism of an antifreeze protein from winter flounder. Nature 375(6530):427–431.
Slocik JM, Wright DW. 2003. Biomimetic mineralization of noble metal nanoclusters. Biomacromolecules 4(5):1135–1141.
Tamerler C, Duman M, Oren EE, Gungormus M, Xiong XR, Kacar T, Parviz BA, Sarikaya M. 2006a. Materials specificity and directed assembly of a gold-binding peptide. Small 2(11):1372–1378.
Tamerler C, Oren EE, Duman M, Venkatasubramanian E, Sarikaya M. 2006b. Adsorption kinetics of an engineered gold binding peptide by surface plasmon resonance spectroscopy and a quartz crystal microbalance. Langmuir 22(18):7712–7718.
Tominaga J, Kamiya N, Doi S, Ichinose H, Maruyama T, Goto M. 2005. Design of a specific peptide tag that affords covalent and site-specific enzyme immobilization catalyzed by microbial transglutaminase. Biomacromolecules 6(4):2299–2304.
Tomizaki KY, Usui K, Mihara H. 2005. Protein-detecting microarrays: Current accomplishments and requirements. Chembiochem 6(5):783–799.
Watzke A, Kohn M, Gutierrez-Rodriguez M, Wacker R, Schroder H, Breinbauer R, Kuhlmann J, Alexandrov K, Niemeyer CM, Goody RS, Waldmann H. 2006. Site-selective protein immobilization by Staudinger ligation. Angew Chem Int Ed 45(9):1408–1412.
Whaley SR, English DS, Hu EL, Barbara PF, Belcher AM. 2000. Selection of peptides with semiconductor binding specificity for directed nanocrystal assembly. Nature 405(6787):665–668.
Willey TM, Vance AL, van Buuren T, Bostedt C, Terminello LJ, Fadley CS. 2005. Rapid degradation of alkanethiol-based self-assembled monolayers on gold in ambient laboratory conditions. Surf Sci 576(1–3):188–196.
Woodbury RG, Wendin C, Clendenning J, Melendez J, Elkind J, Bartholomew D, Brown S, Furlong CE. 1998. Construction of biosensors using a gold-binding polypeptide and a miniature integrated surface plasmon resonance sensor. Biosens Bioelectron 13(10):1117–1126.
Xia YN, Whitesides GM. 1998. Soft lithography. Annu Rev Mater Sci 28: 153–184.
Zhang JK, Cass AEG. 2001. A study of his-tagged alkaline phosphatase immobilization on a nanoporous nickel-titanium dioxide film. Anal Biochem 292(2):307–310.
Zhen GL, Falconnet D, Kuennemann E, Voros J, Spencer ND, Textor M, Zurcher S. 2006. Nitrilotriacetic acid functionalized graft copolymers: A polymeric interface for selective and reversible binding of histidine-tagged proteins. Adv Funct Mater 16(2):243–251.
Zhou HX, Dill KA. 2001. Stabilization of proteins in confined spaces. Biochemistry 40(38):11289–11293.
Zin MT, Ma H, Sarikaya M, Jen AKY. 2005. Assembly of gold nanoparticles using genetically engineered polypeptides. Small 1(7):698–702.