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Immobilization of Enzymes to Silver Island Films for Enhanced Enzymatic Activity

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Abstract

Hypothesis—The performance of the enzyme-based biosensors depends on the enzymatic activity and the use of an appropriate technique for immobilization of enzymes. The incorporation of silver island films (SIFs) into the enzyme-based biosensors is expected to enhance the enzymatic activity and to increase the detectability of analytes of interest.

Experiments—Two enzymes, β -galactosidase (β -Gal) and alkaline phosphatase (AP) were immobilized onto SIFs using the interactions of avidin-modified enzymes with (i) a monolayer of biotinylated bovine serum albumin (b-BSA) and/or (ii) a monolayer of biotinylated poly(ethylene-glycol)-amine (BEA molecular weight: 550 to 10000 Da). To confirm the effect of SIFs on enzymatic activity, two control surfaces (no silver) were also employed.

Findings—No enhancement in enzymatic activity for β -Gal on all SIFs was observed, which was attributed to the inhibition of β -Gal activity due to direct interactions of β -Gal with SIFs. The AP activity on SIFs with BEA was significantly larger than that observed on SIFs with b-BSA, where a 300% increase in AP activity was observed as compared to control surfaces. These observations suggest that SIFs can significantly enhance AP activity, which could help improve the detection limits of ELISAs and immunoassays that employ AP.

Keywords

Silver island films; enzymes; β -galactosidase; alkaline phosphatase; biotin-poly (ethylene-glycol) amine; protein assays; enzymatic activity

Introduction

The specific, selective, and catalytic properties of enzymes have led to their use in diverse applications in biotechnology and biomedical technology. [1] For example, in biosensors, enzymes are employed as recognition and signaling elements for the detection of specific molecular analyte of interest. [2], [3], [4] In this regard, enzymes are immobilized on to surfaces through covalent binding [4], direct crosslinking, [5] and encapsulation [6] of enzymes on different platforms such as alumina, [7] silica, [8] electrode [4] and

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Supporting Information

Contact angle measurements of BEA on SIFs and the colorimetric responses of β -Gal and AP on SIFs.

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nanoparticles. [9] The extent of enzymatic activity after surface immobilization depends on the binding procedure and on the availability of enzymes to substrates.

Since 1990s, plasmonic nanostructures have received increased attention due to their utility in the detection of biomolecular interactions. [10], [11] Salamon et. al. recently demonstrated that plasmonic nanoparticles can be used as a solid-supported planar proteolipid membranes, which can be a good tool for studying the biochemistry and biophysics of membrane-associated receptors and enzymes using surface plasmon resonance (SPR) spectroscopy. [10] Plasmonic nanoparticles have also been used as a platform in the quantitative study of protein-protein interactions with peptides arrays using SPR imaging. [11] In addition, one can create hybrid systems by combining the plasmonic nanoparticles with enzymes, and make use of the dual biological and electronic functions at the same time. Moreover, these hybrid systems can enhance one or both of the functions of its components. For example, Jena et al has demonstrated the use of a highly sensitive nano-architected amperometric sensor based on platinum nanoparticles and enzyme for the detection of hydrogen peroxide, uric acid, cholesterol and glucose [12]. They have found out that by combining nanomaterials and enzymes the analytical performance of their sensor in terms of sensitivity, selectivity, and limit of detection was improved. It was also shown to exhibit a fast and stable response, and did not undergo deactivation as compared to the unmodified sensors. In another study, Kirchhoff et al has studied the electrodeposition of colloidal gold nanoparticles on gold electrodes for the attachment of acetylcholinesterase, which was then used in the electrochemical detection of thiocholine. [13] Gold nanoparticles on gold electrodes were found to enhance the adsorption and stability of acetylcholinesterase, making it highly sensitive and selective in the detection of thiocholine and acetylcholinesterase inhibitors at low inhibitor concentrations while maintaining the performance of the enzyme upon immobilization for up to 1 week. However, a significant decrease in sensor response was observed in the absence of the nanoparticle layer. [13]. Most recently, Jia and co-workers has described the detection of carcinoembryonic antigen [14], using enzyme-labeled gold nanoparticle probes. Gold nanoparticle probes were developed by binding gold nanoparticles with a detection antibody, single-stranded DNA, and streptavidin-HRP, which was then immobilized onto a magnetic microparticle probe that contains a capture antibody. Their results showed an improvement in detection limit with high sensitivity and specificity than the conventional enzyme-linked immunosorbent assay (ELISA).

The Aslan Research Group has recently demonstrated the combined use of plasmonic nanoparticles, i.e., SIFs, with horse radish peroxidase (HRP) to increase the HRP activity in a biosensing scheme. [12] in this work, three different SIFs with different extent of loading on the glass slide (based on the SPR peak at 420 nm for silver: low loading: $A=0.38$, medium loading: $A=0.55$ and high loading: $A=1.1$) and four different enzyme immobilization strategies: i) a biotin-avidin protein assay: (for protein assays), ii) self-assembled monolayer of hexamethylene diamine: (for covalent binding of enzymes), iii) poly-L-lysine layer: (for covalent binding of enzymes), and iv) Biotin-Poly (Ethylene-glycol)-Amine (BEA): (for protein assays) were used. [13] This work enabled us to investigate the effect of enzyme immobilization on the enzymatic activity on SIFs. The comparison of the enzymatic activity on SIFs using all four immobilization strategies demonstrated that an increase of ~300% in enzymatic conversion of organic substrate by HRP was observed from SIFs with high using only strategies (i) and (iv). [13] These results also provided direct evidence that the enzymatic activity is affected by the enzyme-nanoparticle distance and the extent of loading of silver nanoparticles.

In this study, we investigated the use of SIFs with other enzymes, namely alkaline phosphatase (AP) and β -Galactosidase (β -Gal). AP and β -Gal are typically used in

conjunction with a biotinylated antibody or avidin (in excess) and a substrate for the detection of an antigen. [14] Recently, AP was used in an enzyme-based amplification to demonstrate the electrochemical sensing of DNA sequences, [15] in the detection of glucose and in the determination of cell growth using an acid assay. [16], [17] β -Gal is a well-known enzyme that hydrolyses lactose into its monosaccharide units of glucose and galactose, which has been used in biosensors. [18, 19] SIFs were chemically deposited on to silanized glass slides with different extent of surface loading. In addition, two control surfaces were also employed to investigate the effect of plasmonic surfaces on the enzyme activity: 1) nano-glass beads ($\sim 22 \pm 3.5$ nm) on glass slides were used as a platform for the quantitative comparison of protein surface coverage onto roughened surface similar to that SIFs generates on glass slides and 2) blank glass slides. In a previous publication, [20] our laboratory reported that a similar protein (b-BSA) surface coverage is obtained on SIFs and blank glass slide. [20]

We observed a significant increase in AP activity on all BEA-modified SIFs, where the largest increase ($\sim 300\%$) in AP activity was observed on SIFs (high loading) using BEA-5000 Da, as compared to the two control surfaces. Since the extent of enzyme on all surfaces was comparable and similar chemical environment was maintained on all surfaces, the increase in AP activity is directly attributed to the SIFs. It was also found that the loss of SIFs from the glass surface was negligible when BEA is used to immobilize AP. On the other hand, no enhancement was observed for β -Gal on all SIFs, where the loss of SIFs from the surface was significant. These results showed that SIFs can be incorporated into the current enzyme-based biosensing applications for the detection of biotinylated compounds, where the observed colorimetric response is significantly enhanced. The enhancement of colorimetric response has implications in increased detectability of a target biomolecule.

Materials and Methods

Materials

Sodium hydroxide (anhydrous, 98%), D-glucose, silver nitrate, ammonium hydroxide (30%), sulfuric acid (A.C.S reagent grade), 3-aminopropyltriethoxysilane (APS) (99%), hydrogen peroxide (30 wt. % solution in water), albumin from bovine serum (99%) (BSA), albumin biotin labeled bovine (b-BSA), alkaline phosphatase yellow (pNPP) liquid substrate system for ELISA, avidin-alkaline phosphatase from streptomyces avidinii (AP-avidin), and 2-nitrophenyl β -D-galactopyranoside (ONPG) were all obtained from Sigma-Aldrich. Avidin- β -galactosidase conjugate (β -Gal-avidin) was purchased from Invitrogen (USA). Sodium phosphate dibasic and monobasic anhydrous, Potassium phosphate dibasic, 2-Mercaptoethanol, sodium carbonate, magnesium chloride (A.C.S certified) was purchased from Fisher Scientific. Citric acid (analytical reagent grade) was purchased from Mallinckrodt Chemical Works, and sulfuric acid (A.C.S reagent grade), and ethyl alcohol (200 proof) were obtained from PHARMCO. Tri(hydroxymethyl) aminomethane was purchased from Research Organics, Inc. Glass slides (micro slides, thickness: 0.96 to 1.06 mm) used in this work was obtained from Corning Inc. Silicone isolator adhesive (2.0 mm deep and 4.5 mm diameter) were all obtained from Electron Microscopy Sciences (Hatfield, PA). Microtest plate 96 well high throughput screening (HTS) plates were obtained from Sarstedt, Inc., and biotin-polyethylene glycol amine (BEA) was brought from Layson Bio, Inc. All reagents were used as received. Nano-glass beads (22 ± 3.5 nm) were purchased from Microsphere-Nanospheres Inc. Triethoxysilylbutyraldehyde and bis(trimethoxysilyl) octanes were bought from Gelest, Inc. All aqueous solutions were prepared using deionized water (> 18.0 M Ω •cm resistivity at 25 °C) obtained from Millipore Direct Q3 system.

Methods

(1) Deposition of Silver Island Films (SIFs) onto APS-coated glass slides—The silanization of the glass slides and deposition of SIFs were performed using previously described procedures. [12] The extent of SIFs on glass slides were varied by keeping the glass slides in the solution at different time intervals; low loading (for ~50 seconds), medium loading (for ~2 minutes), and high loading (for ~4 minutes). A silicone isolator (2.0 mm deep and 4.5 mm diameter) was used to cover SIFs, before proceeding to the next steps in this work.

(2) Surface Modification of Glass Slides using Organosilanes (A Control Surface)—We adopted a previously described procedure for the surface modification of glass slides using an organosilane. [21] In this regard, an organosilane (i.e., triethoxysilylbutyraldehyde) solution was prepared using ethanol 95%, water 3%, and organosilane 2% all in a closed glass bottle containing a magnetic stirrer. The mixed solution was allowed to sit for 5 minutes before transferring it into a closed vial, which the clean glass slides were immersed. [Note: *The glass slides were cleaned using piranha solution (H_2SO_4 : H_2O_2) (3:1). Piranha solution is extremely dangerous and must be handled with care*]. The glass slides were then incubated in a closed vial containing 50 mL of the prepared organosilane solution, which was allowed to incubate for 30 minutes. The slides were later rinsed with ethanol to remove the unbounded materials and dried using air. Annealing of the silanes was achieved by placing the slides in the oven for 30 minutes at 100°, before use.

(3) Immobilization of Nano-glass Beads onto Silanized Glass Slides (A Control Surface)—Nano-glass beads ($\sim 22 \pm 3.5$ nm) was purchased from the manufacturer as a dispersion (nano-glass beads: 50% and water 50%). The nano-glass beads were separated from water using a centrifuge, before proceeding with washing the beads with piranha solution. The beads were then re-suspended using ethanol before immobilization onto silanized glass slides. Immobilization of the nano-glass beads onto the glass slides was performed by means of: (i) silanization of the glass slides with bis(trimethoxysilyl) octane, this bis-silane structure has two terminal silane groups which can afford for the immobilization of nano-glass beads onto glass slides. Bis-silane was then prepared using ethanol 95% and bis (trimethoxysilyl) octane 5%. Silanization was performed as previously described procedure above, (ii) nano-glass beads were then immobilized on to the silanized glass via cross linking of the silane group and nano-glass beads. Immobilized nano-glass beads on glass slides were then washed with ethanol to remove the unbounded materials. The surface was then coated with triethoxysilylbutyraldehyde before use.

(4) Biotin-Avidin Protein Assay for the Immobilization of Enzymes onto SIFs—Schematic representation for the preparation of biotinylated-BSA assay is shown in scheme 1. Biotinylated-BSA was attached to SIFs [12] before the binding of AP-avidin and β -Gal-avidin on the surface of SIFs via specific interaction between biotin-avidin groups. An AP-avidin stock solution (1.0 mg mL^{-1}) was prepared using Tris buffer containing 1.0 mM of magnesium chloride at pH 8, the solution is then diluted 1:10 to obtain a final concentration of 0.1 mg mL^{-1} . β -Gal-avidin stock solution (2.0 mg mL^{-1}) was prepared using 100.0 mM potassium phosphate containing 1.0 mM magnesium chloride and 0.1 M 2-mercaptoethanol at pH 7, the solution is then diluted 1:10 to obtain a final concentration of 0.2 mg mL^{-1} . We note that these values correspond to the concentration of enzymes used in a typical ELISA. The prepared enzyme solutions ($\sim 30 \mu\text{L}$) with final concentrations mentioned above were added into the silicone isolator chambers, and were allowed to incubate for 30 minutes at room temperature. SIFs were then washed using deionized water and dried with air to

remove the unbounded material. Enzymatic activity was carried out using the substrates pNPP for alkaline phosphatase and ONPG for β -galactosidase, respectively.

(5) Preparation of Biotin-Poly (Ethylene-glycol) Amine (BEA) Assay on SIFs-Deposited Glass Slides, Silanized Glass, and Nano-Glass Beads on Glass—

Schematic representation of BEA assay is shown in scheme 2. SIFs were modified with BEA as previously described. [13] The prepared enzyme solutions ($\sim 30 \mu\text{L}$) with final concentrations mentioned above were added into the silicone isolator chambers, and were allowed to incubate for 30 minutes at room temperature. In some experiments, to determine the extent of enzyme adsorption on all surfaces, the absorption of the enzyme solutions was measured before their addition on to the surfaces and after the incubation process. The difference in absorption peak at 280 nm, specific to enzymes and proteins, were used to assess the extent of enzyme adsorption onto the surfaces. The surface coverage of enzymes was determined by converting the absorption at 280 nm to mass of enzyme adsorbed per area of each well. The chambers were washed using deionized water, and then dried with air to remove the unbounded material. Enzymatic activity was carried out using the substrates pNPP for alkaline phosphatase and ONPG for β -galactosidase, respectively.

(6) Preparation of pNPP and ONPG Solutions—The pNPP solution was used directly from the alkaline phosphate yellow substrate obtained from Sigma-Aldrich without any further dilution. The corresponding wavelength of the product (*p*-nitrophenol) obtained after interaction of pNPP with AP-avidin was at 405 nm in all experiments. ONPG stock solution (20.50 mg mL^{-1}) was prepared using 100 mM sodium phosphate buffer at pH 7.3. The solution was then completely dissolved using a sonicator. The wavelength obtained from the product (*o*-nitrophenol) after interaction of ONPG with β -Gal-avidin was at 430 nm.

(7) Enzymatic Activity Measurements—The conversion of pNPP by AP produced a colored product (*p*-nitrophenol) from colorless to yellow. The colored product ($30 \mu\text{L}$) was then taken from the SIFs surface after 2 minutes of incubation and placed in each HTS well containing the stopping reagent [3.0 M of sodium hydroxide ($200 \mu\text{L}$)] to stop the conversion of pNPP followed by measuring absorption spectrum of the initial solution (pNPP), final solution (colored product) and control solution. Control experiment, where the BEA and b-BSA were omitted from the surface was used to measure the extent of absorbance due to the non-specific binding of the enzymes onto SIFs. [Note: it is important to state that the use of silicon isolator afforded for the multiple bioassays of repeated samples to be carried out in a confined volume of $30 \mu\text{L}$ (volume of each well) on the same SIFs surface]. [22]

(8) Water Contact Angle on SIFs—The contact angle of water ($20 \mu\text{L}$) on non-modified SIFs and modified SIFs surfaces using BEA and b-BSA, silanized glass, and silanized nano-glass beads on glass was measured using Kruss Drop Shape Analysis System (DSA) Model Number 10 MK2.

Other Instrumentation

In this work, all absorption measurements were performed using Cary 50 Bio UV-Spectrophotometer obtained from Varian, Inc. Real-color photographs of SIFs was taken by a 5 MP digital camera.

Results and Discussion

Surface modification of silver nanostructures with proteins and other $-\text{NH}_2$ containing compounds (such as BEA) occurs via the interactions of these groups onto silver surface.

[23] To confirm that the surface modification of SIFs was carried out as intended, the contact angle of water was measured on the different SIFs surfaces (low, medium and high loading), and for the sake of brevity, these results were provided in the Supporting Information. Subsequently, we investigated whether the enzymatic activity of β -Gal and AP can be enhanced by SIFs. These enzymes were placed away from the silver surface using biotin-avidin protein assay and/or biotinylated BEA (with different PEG chain lengths). The reasons for selecting these methods were: (i) BEA has a poly (ethylene-glycol) spacer arm, which places the enzyme away from the silver surface. [13] [Note: Poly (ethylene-glycol) spacer arm contained in BEA compounds is known for their high hydrophilicity and chain length flexibility. [24] [25] PEG with a molecular weight of 5000 Da has been revealed to be most effective in maximizing the suppression of non-specific adsorption of protein onto glass substrate] [24]–[25], (ii) biotin-avidin protein assay places the enzyme ~4–8 nm away from the silver surface and was also proven to be an effective strategy. [12] Based on our previous observations on the lack of enhancement provided by self-assembled monolayer of hexamethylene diamine and poly-L-lysine layer, we did not attempt to employ them here.

We first investigated the effect of the extent of loading of SIFs on glass slides and the two enzyme immobilization strategies mentioned above on the enzymatic activity. Figure 1 shows the summary of the result obtained after the conversion of ONPG to a colored product by β -Gal on SIFs and on silanized glass slides (No SIFs, a control experiment) using b-BSA and BEA. As shown in Figure 1A, the absorption value of colored product at 425 nm on glass slides is significantly larger than those measured from SIFs (low, medium, and high loading) using both b-BSA and BEA. The observation of lower enzymatic activity of β -Gal on SIFs than on glass slides can be partially attributed to the fact that β -Gal contains between 12–20 free sulfhydryl (-SH) groups: these -SH groups are susceptible to reacting with metal ions including silver, copper, cadmium, iron, and other metals, which are known to inhibit enzyme activity. [26], [27] Our efforts in placing the enzyme ~6 nm BEA-3400 Da and ~4–8 nm (b-BSA) away from the silver surface did not improve the enzyme activity. We hypothesize that β -Gal interacted with SIFs on surfaces not protected by BEA or b-BSA and/or by replacing the primary -NH₂ groups of BEA. It should be noted that neither HRP nor AP has any free sulfhydryl groups, which helps in their resistance to metal-mediated inhibition. In control experiments, where BEA-3400 Da and b-BSA were omitted from the assay, no enzymatic activity was observed (Figure 1C). The real-color photographs (Figure 1B-inset) corroborate the results obtained by absorption spectroscopy. Figure 1D shows the overall comparison of the absorption values at 445 nm for b-BSA and BEA-3400 Da and control experiments.

To ascertain whether a potential loss of SIFs from the surface also contributed to the observed decrease in enzymatic activity on SIFs, we investigated the extent of SIFs before and after their use in enzymatic reactions by measuring the variations in the SPR peak of SIFs and comparing the surfaces using real-color photographs. The real-color photographs of SIFs before use and SIFs surfaces with β -Gal using b-BSA and BEA (Figure 2A) visually show a loss of SIFs from the surfaces. This observation is corroborated with the corresponding SPR peaks for SIFs as shown in Figure 2B. The observed decrease in the SPR peak for SIFs with low (<60%), medium (<65%) and high deposition (<70%), implies that β -Gal and SIFs were removed from the surface and this loss contributed to the lower enzymatic activity observed on SIFs as compared to glass slides.

Figure 3 shows the summary of the result obtained after the conversion of pNPP to a colored product by AP deposited onto SIFs and silanized glass slides (No SIFs, a control experiment) using b-BSA. As shown in Figure 3A, the absorption spectrum of colored product on SIFs (low, medium, and high loading) using b-BSA shows an increase in the peak absorption values at 405 nm as the loading of SIFs is increased, as compared to the

colored product obtained on glass slides. A significant increase in the absorption of the colored product on SIFs at 405 nm using BEA-3400 Da was also observed (Figure 3B), as compared to the control experiment (no SIFs, no BEA or b-BSA Figure 3C). Real-color photographs of colored solution placed in HTS wells after the completion of enzymatic reaction on SIFs (low, medium, and high loading) using b-BSA and BEA-3400 Da corroborate the variation in absorbance values observed in the absorption spectrum: the color of the solution is darker as the loading of SIFs is increased (Figure 3A and 3B-inset). In control experiments (Figure 3C), where BEA and b-BSA were omitted from the surface, the absorption spectrum shows a decrease in enzymatic activity on SIFs as compared to BEA-3400 Da and similar values to those observed using b-BSA.

To effectively assess all the results, the overall comparison of the absorption values at 405 nm for b-BSA and BEA-3400 Da and control experiments are shown in Figure 3D. When immobilized using b-BSA, the enzymatic activity of AP on SIFs is clearly higher than those on glass slides. Based on our previous work, where we showed that the extent of proteins on SIFs (with medium loading) and glass slides were similar, [28] one can expect the absorbance values for the colored product for these two surfaces (Figure 3A: lines 1 and 3), to be similar. However, SIFs display a high background signal using b-BSA (control, no b-BSA), which implies that the observed increase in enzymatic activity in b-BSA is due to AP adsorbed on SIFs in a non-specific manner. These observations provide a direct evidence for the increase in AP activity on SIFs (medium loading) is due to the presence of SIFs.

When BEA-3400 Da was employed, the enzymatic activity of AP on SIFs is significantly higher than those on glass slides and the background (control, no BEA). These observations imply that the immobilization of AP onto SIFs using a monolayer of BEA-3400 Da, which positions the enzyme up to ~6 nm (with avidin) away from the silver surface for increased enzymatic activity. [13] The overall increase in enzymatic activity can be partially attributed to the nature of BEA and the use of avidin, which exposes AP to the substrate present in bulk more efficiently than biotinylated BSA. The observed increase in AP activity as the loading of SIFs is increased can be attributed to the availability of more AP on the surface due to the increased number of SIFs, where BEA can be adsorbed. It should be noted that the experimental design and the data presented in Figures 1–4 were used to compare the two enzyme immobilization procedures. In order to demonstrate the effect of SIFs on the enzyme activity while maintaining the same chemical environment, we have varied the chain length of BEA. These results are discussed later in the text.

It is also important to comment on the differences in the absorption spectrum observed for both strategies: an increase in the absorption values < 400 nm is observed using b-BSA. This could be due to two possible phenomena: 1) due to the release of b-BSA in to the colored product during the enzymatic activity, 2) due to the presence of silver nanoparticles in to the colored product. Since BSA absorb light at 280–290 nm region, we hypothesize that the increase in the absorption values < 400 nm may be due to loss of SIFs from the surface. In this regard, we investigated the extent of SIFs before and after their use in enzymatic reactions by measuring the variations in the SPR peak of SIFs and comparing the surfaces using real-color photographs.

Figure 4 shows the real-color photographs of SIFs before use and after use when employing b-BSA and BEA-3400 Da with AP and the corresponding SPR peak values of SIFs. The real-color photographs of SIFs after their use with b-BSA visually shows a loss of SIFs from the surface (Figure 4A). On the other hand, virtually no loss from SIFs after using BEA-3400 Da was observed (Figure 4A). These visual observations were corroborated by SPR peak measurements as shown in Figure 4B. These results imply that the increase in the absorption values < 400 nm using b-BSA is most likely due to the loss of SIFs from the

surface, which was in accordance with our previously published results. [12], [13] Figures 3D and 4B demonstrate that the use of BEA and avidin as linkers for the immobilization of AP onto SIFs is efficient in yielding increased enzymatic activity without the loss of SIFs and AP from the surface.

To investigate whether the variation in the distance between the enzyme and the surfaces by increasing the chain length of BEA, while maintaining similar chemical environment for the enzymes, can improve the enzyme activity we have carried out additional experiments with SIFs and the two control surfaces. Since BEA does not bind to glass slides or glass nano-beads, we modified the glass with an organosilane (triethoxysilylbutyraldehyde) that has terminal a $-CHO$ group that couples to the terminal $-NH_2$ group of BEA via Schiff reaction. BEA compounds used in this work contained PEG groups that were previously studied (in separate papers for other purposes than reported in this work); PEG-2000 Da (~3.5 nm in length), [29] PEG-3400 Da (~6 nm), [13] PEG-5000 Da (~10 nm). [29] When used as a linker, PEG-5000 Da was shown to provide homogeneous surfaces with high resistance to non-specific binding of proteins. This was attributed to the formation of mushroom-like monolayer on the glass surface by PEG-5000 Da. [30] PEG-2000 Da was shown to be less homogeneous and less resistant to non-specific adsorption. [29] In addition, PEG-3400 Da was shown to enhance chemical stability of protein and also increase the stability of immobilized antibodies. [31] It was also shown that PEG-750 Da forms a short, brush like monolayer on the surface of glass. [30] In this regard, the presence of BEA with longer chain length on SIFs is thought to reduce the interaction of $-SH$ groups of enzymes (particularly for β -Gal) with SIFs. The choice of using SIFs with high loading was based on the results shown in Figures 1–4, where the largest extent of enzyme activity was obtained using these SIFs. The use of nano-glass beads on glass as a control surface afforded for the creation of a roughened surface similar to SIFs (but without the presence of plasmons), where the extent of protein coverage on both surfaces are comparable.

Figures 5 and 6 show the summary of the results obtained for i) maximum enzymatic activity, ii) adsorbed enzyme (i.e., enzyme coverage on the surface of the different surfaces), and iii) the activity without enzyme, which were plotted against the molecular weights of the different BEA immobilized on to SIFs, silanized nano-glass beads on glass, and silanized glass. In addition, enzyme activities on all surfaces without the presence of enzyme (a control experiment) were also measured.

Figure 5 shows that the extent of surface coverage of AP on all surfaces modified with BEA were similar and were increased as the molecular weight of BEA was increased. The surface coverage of AP was leveled off after BEA-3400 Da to BEA-5000 Da, where the maximum surface capacity for the immobilization of AP was reached. As expected, AP activities on all surfaces showed direct correlation with the observed trend in adsorbed AP. However, there were stark differences in the extent of AP activity on SIFs as compared to the two control surfaces (Figures 5 and S1-Supporting Information). Although the extent of AP adsorption was similar (in a similar chemical environment) on all surfaces, AP activity was significantly larger on SIFs than the other two control surfaces when BEA with molecular weight was > 3400 Da. In addition, the loss of enzymes on BEA-modified SIFs (BEA-3400, 5000, 10000 Da) was minimal (Figure S3, Supporting Information). These observations provide a direct evidence for enhancement of AP activity by SIFs.

It is also important to investigate whether an increase in the distance between the β -Gal and the SIFs reduces the interaction of $-SH$ groups of β -Gal with SIFs and the activity of β -Gal can be enhanced. Figure 6 and Figure S2 (Supporting Information) show the summary of the results for β -Gal with SIFs (high loading-Figure 6A), silanized nano-glass beads on glass (Figure 6B), and silanized glass Figure 6C. Despite the similarities in the extent of adsorbed

β -Gal on all surfaces, β -Gal activity on SIFs was significantly lower than on the two control surfaces. We hypothesize from this result that BEA that has a longer polymer chain on SIFs were unable to reduce the β -Gal interaction with SIFs. We also hypothesize that BEA monolayers on SIFs brings the enzyme in close proximity with the metal surface, thereby increasing the interaction between the $-SH$ and silver. This hypothesis is based on the water contact measurements shown in Figure S2 (Supporting Information), where the water contact angle for water was significantly reduced on BEA-modified SIFs that results in the reduction of the surface tension of the solution containing β -Gal on SIFs. On the other hand, the water contact angle for the solution containing β -Gal on the two control surfaces were higher and since there are no silver nanostructures on these surfaces, the activity of β -Gal was higher as compared to SIFs. In addition, the loss of β -Gal from SIFs contributed to the lack of enzyme activity. Subsequently, we conclude that the use of BEA is not suitable for applications that employ β -Gal on SIFs.

To quantitate the effect of SIFs on enzyme activity as compared to the two control surfaces, the results obtained using BEA-5000 Da is further investigated and summarized in Table 1. BEA-5000 Da was selected due to the largest observed AP activity employed with SIFs (high loading), as shown in Figure 5. The extent of AP and β -Gal coverage on all surfaces were comparable; i) AP: SIFs on glass (high loading) (23 ± 2.0 ng/mm²), silanized nano-glass beads on glass (29 ± 5.3 ng/mm²), and silanized glass (19 ± 0.6 ng/mm²), ii) β -Gal [SIFs on glass (high loading) (23 ± 0.5 ng/mm²), nano-glass beads on glass (25 ± 4.2 ng/mm²), and silanized glass (16 ± 6.0 ng/mm²)]. The net enzyme activity for AP and β -Gal was calculated as follows: [Observed enzyme activity – (Non-specific enzyme activity + background absorption)]. These calculations show that the net AP activity on SIFs (while maintaining similar chemical environments) is ~3-fold and 1.5-fold larger than on the nano-glass beads and on silanized glass. Enzymatic activity of β -Gal on the surface of nano-glass beads on glass and silanized glass slides (No-SIFs, control experiment) were larger than on SIFs as discussed previously in the text. These calculations were also done for all surfaces modified with other BEA compounds (Table S3, Supporting Information), which shows that BEA compounds with molecular weight > 3400 Da-10000 Da immobilized on SIFs can be used for an enhanced enzymatic activity. Work is currently underway for the use of other enzymes with SIFs and will be reported in due course.

Conclusions

In this work, we investigated the effect of two immobilization strategies for enhanced enzymatic activity using SIFs. The activity of enzymes on SIFs with different surface loading and on two control surfaces (nano-glass beads and silanized glass slides) were assessed by colorimetric response of the reactions between AP with pNPP, and β -Gal with ONPG, respectively. The study revealed that the activity of β -Gal was inhibited due to its interactions with SIFs, which results in no colored product formation after enzymatic activity. The use of AP on SIFs showed an increased formation of colored product after enzymatic activity, which was further increased as the loading of SIFs was increased. In addition, an insignificant amount of b-BSA and SIFs was lost from the surface. The variation of the distance between the enzymes and SIFs, while maintaining similar chemical environments to improve the enzyme activity resulted in a further enhancement of AP activity. However, the activity of β -Gal on SIFs was not improved by the increase in the distance between β -Gal and SIFs. These observations suggest that SIFs can significantly enhance AP activity, which could help improve the detection limits of ELISA and immunoassays that employ AP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. The effect of silver islands films on enzyme activity
2. Two immobilization strategies
3. Enhanced alkaline phosphatase activity on SIFs
4. No enhancement of β -Gal activity due to inhibition by SIFs

Figure 1.

Absorption spectrum (A) of ONPG on SIFs (low, medium, and high loading) using biotin-BSA (B) and BEA-3400 Da. For comparison purposes, absorption spectrum of ONPG in solution containing β -Gal was also shown (red line). Inset- Real-color photographs of ONPG in HTS wells after enzymatic reaction on SIFs: A and B represents biotin-BSA and BEA-3400 Da. C and D represents ONPG in HTS wells after enzymatic reaction on glass and control experiment on glass, respectively. (C) Absorption spectrum of control samples with β -Gal and (D) Comparison of absorbance values at 425 nm.

Figure 2.
 (A) Real-color photographs of SIFs before use and after using biotin-BSA and BEA-3400 Da with β -Gal: low, medium and high loading. (B) Corresponding surface plasmon resonance peak values of SIFs.

Figure 3.

Absorption spectrum of pNPP on SIFs (low, medium and high loading) using (A) biotin-BSA (B) BEA-3400 Da and (C) Control experiments for AP. (D) Comparison of absorbance values at 405 nm for AP. Glass: has no SIFs.

Figure 4.

(A) Real-color photographs of SIFs before use and after using biotin-BSA and BEA-3400 Da with AP: low, medium and high loading. (B) Corresponding surface plasmon resonance peak values of SIFs.

Figure 5.

Plots of maximum AP activity w/BEA, and activity w/o AP versus molecular weight of different BEA on different platforms (A) SIFs, (B) Nano-glass beads on glass, and (C) Silanized glass. D shows the adsorbed AP on the different platforms.

Figure 6.

Plots of maximum β -Gal activity w/BEA, and activity w/o BEA versus molecular weight of different BEA on different platforms (A) SIFs, (B) Nano-glass beads on glass, and (C) Silanized glass. D shows the adsorbed β -Gal on the different platforms.

Biotin-BSA **Scheme 1.**

Schematic depiction of immobilization strategies for AP and β -Gal: Enzymes are deposited onto SIFs using Biotin-BSA-avidin interactions. Glass slides were modified with 3-aminopropyltriethoxysilane, which contains terminal amine groups. Biotin-BSA-avidin was omitted in the control samples. The inset also applies to scheme 2.

Biotin-PEG-Amine **Scheme 2.**

Schematic depiction of immobilization strategies for AP and β -Gal: Enzymes are deposited onto Silanized glass, Silanized nano-glass beads, and SIFs using Biotin-PEG-avidin interactions. Biotin-PEG-Amine and Enzymes were omitted in the control samples.

Table 1

Summary of results of using AP and β -Gal using BEA-5000 Da as the linker.

BEA 5000 Da		SIFs on glass (high loading)	Nano-glass beads on glass	Silanized glass
Adsorbed Enzyme (ng/mm ²)	AP	23 $\pm$ 2.0	29 $\pm$ 5.3	19 $\pm$ 0.6
	β -gal	23 $\pm$ 0.5	25 $\pm$ 4.2	16 $\pm$ 6.0
Enzyme Activity (Abs)	AP	1.0 $\pm$ 0.02	0.51 $\pm$ 0.02	0.32 $\pm$ 0.01
	β -gal	0.005 $\pm$ 5 $\times 10^{-4}$	0.28 $\pm$ 0.023	0.37 $\pm$ 0.011
Non-Specific Enzyme Activity (Abs)	AP	0.26 $\pm$ 0.05	0.14 $\pm$ 0.005	0.07 $\pm$ 0.004
	β -gal	0.005 $\pm$ 5 $\times 10^{-4}$	0.012 $\pm$ 3 $\times 10^{-4}$	0.003 $\pm$ 3 $\times 10^{-4}$
Background (Abs)	AP	0.016 $\pm$ 0.003	0.002 $\pm$ 0.001	0.003 $\pm$ 0.001
	β -gal	0.001 $\pm$ 3 $\times 10^{-4}$	0.003 $\pm$ 4 $\times 10^{-4}$	0.003 $\pm$ 7 $\times 10^{-4}$
Net Enzyme Activity (Abs)	AP	0.724	0.362	0.245
	β -gal	0.000	0.269	0.364
Enhancement Factor (Abs on surface/Abs on glass)	AP	3.0	1.5	-
	β -gal	0	0.74	-