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Enhancement of Enzymatic Colorimetric Response by Silver Island Films on High Throughput Screening Microplates

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Abstract

In this study, we report the use of an enzyme-based hybrid platform, which is comprised of silver island films, enzymes (HRP and AP) and high-throughput screening (HTS) microplates, to enhance the colorimetric response of enzymatic reactions. The hybrid platform was designed in a two-step process: (i) deposition of SIFs onto HTS microplates with low, medium, and high loading (refers to the extent of the surface plasmon resonance peak of SIFs at 460 nm) using Tollen's reaction scheme; and (ii) attachment of b-BSA or BEA as linkers for the immobilization of enzymes. The presence of SIFs within the wells of the HTS microplates was confirmed using an optical spectrophotometer and real-color photography. Control experiments, where SIFs were omitted from the surfaces were carried out to confirm the effect of SIFs on the enzymatic colorimetric response. Significant colorimetric signal enhancement was observed for HRP or AP on SIFs (high loading) deposited HTS microplates using b-BSA (up to ~ 3-fold for AP and ~6-fold HRP) or BEA (up to ~ 7-fold for both HRP and AP), as compared to our control samples. The observed increase in colorimetric response can be attributed to the nature of BEA, which exposes surface-bound enzymes to the substrate present in bulk more efficiently than b-BSA. This study proves that SIFs can serve as a valuable tool to improve the signal output of existing bioassays carried out in HTS microplates, which can be applicable to the field biosensors and plasmonics.

Keywords

Plasmonic nanoparticles; silver island films; high-throughput screening microplates; horseradish peroxidase; alkaline phosphatase; biotin-poly(ethylene-glycol) amine

1. Introduction

The design and application of enzyme-based bioassays using planar solid platforms involves the successful immobilization of enzymes onto the solid surface. The basic requirements for enzyme immobilization onto any solid platform includes; (i) biocompatibility with the

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enzyme, (ii) availability of reactive functional groups necessary for enzyme attachment, (iii) enzyme stability, (iv) use of no toxic reagents, and (v) allow selective and sensitive for the detection of target analytes. The control over these requirements is critical to achieve a stable, catalytically active enzyme layer.

In the last two decades, nanostructured materials have received increased attention due to their utility in the detection of biomolecular interactions.(Salamon et al., 1997),(Wegner et al., 2002) In this regard, numerous researchers have contributed to the development of enzyme/nanoparticle hybrid systems that exploit the use of plasmonic (and other) nanoparticles on planar substrates such as glass,(Karakouz et al., 2011) plastic,(De et al., 2008),(Fan et al., 2010) and electrodes.(Lisa et al., 2009),(Cui et al., 2008) Glass substrates are the commonly used planar surfaces due to their low cost and defined surface chemistry. However, poor adhesion of plasmonic nanoparticles onto glass surfaces could result in enzyme instability, and subsequently in low signal output.(Karakouz et al., 2011) Plastics are employed as solid platforms as an alternative to glass substrates.(De et al., 2008) HTS microplates are based on plastics and are widely employed in laboratory applications for the detection of analytes of interest multiplexed and high throughout fashion.

One can find a plethora of reports on the use of nanostructured materials as a biosensing tool in literature. For example, Feng et. al. has studied the sensing of a model analyte (i.e., glucose) using nanoparticles (i.e., plasmonic interferometer).(Feng et al., 2011) The plasmonic interferometer was designed and fabricated using groove-slit-groove structures in a 300 nm thick silver film. In order to assess the sensing scheme, various concentrations of glucose solutions in water were allowed to flow in and out of the micro channel of the grooves, and the transmitted light intensity was then observed for each plasmonic interferometer.(Feng et al., 2011) Their results prove that plasmonic interferometry is a novel approach for the design of a HTS platform for measuring biological materials rapidly with very high sensitivity.(Feng et al., 2011) In another study, nanostructured materials were employed for the construction of a plasmonic fluorescent nanocomposites, in which HTS platforms were used to characterize the spectral properties, aggregation kinetics, aggregation density, and photostability of the nanocomposites.(Achyuthan et al., 2012) It was reported that HTS platform afforded for the preparation of a novel enzyme assay using nanocomposites for signaling catalytic reactions.(Achyuthan et al., 2012)

More recently, Abel et. al. has demonstrated an immobilization strategy that could lead to the improvement of enzyme and platform stability, and increase the efficiency of enzymatic reactions on solid platforms.(Abel and Aslan, 2014) This technique employs a hybrid system that comprises of plasmonic nanoparticles (i.e., SIFs), immobilizers (i.e., biotinylated protein and a biotinylated polymer (average molecular weight of 550–10,000 Da), enzymes (i.e., HRP or AP or β -Gal), and solid platform (i.e., microscopic glass slides). Our research group have also carried out fundamental studies on the effects of the nanostructured materials, other linkers, and enzymes in the design of the enzyme-based biosensors on glass substrates.(Abel and Aslan, 2012),(Abel et al., 2011) These studies afforded us to expand our research in to other solid platforms, such as HTS microplates, which can be applicable to multiplexed chemical and biological sensing. Our methodology affords for the use of

commercially available HTS microplates and that takes advantage of the combined use of plasmonic nanoparticles with enzymes to fabricate a multifunctional hybrid system.

In this study, we present the surface modification of commercially available HTS microplates with SIFs and the application of these SIFs-modified HTS microplates in the enhancement of colorimetric response of enzymatic reactions. Scheme 1 shows the depiction of two different methodologies for the immobilization of enzymes (HRP and AP) onto the SIFs-modified HTS microplates that was carried out using b-BSA or BEA linkers. SIFs were deposited at different extents of loading (i.e., low, medium, and high) within the wells of the HTS microplates. We also note that the extent of loading of SIFs refers to the surface plasmon resonance peak of silver at 460 nm, i.e. low loading: Absorbance (A)_{460 nm} = ~0.20, medium loading: $A_{460 \text{ nm}}$ = ~0.30, high loading: $A_{460 \text{ nm}}$ = ~0.50. The colorimetric response of HRP and AP were assessed by transferring the colored product at the end of the enzymatic reaction (~2 min) in to another HTS microplate (that do not contain SIFs) with a stopping reagent (sulfuric acid for HRP and sodium hydroxide for AP) and measuring the absorption spectrum (380–600 nm). The characteristic peaks for the colored product generated by HRP and AP occurs at 495 nm and 405 nm, respectively. Control experiments, where SIFs were omitted from the surface, were carried out to determine the effect of SIFs on the colorimetric response.

2. Materials and Methods

2.1. Materials

Hydrogen peroxide (30 wt. % solution in water), ammonium hydroxide (30%), nitric acid (A.C.S reagent grade), silver nitrate (A.C.S reagent grade), D-glucose, streptavidin-peroxidase from *Streptomyces avidinii* (HRP-streptavidin), streptavidin-alkaline phosphatase from *Streptomyces avidinii* (AP-streptavidin), alkaline phosphatase yellow (pNPP) liquid substrate system for ELISA, sodium hydroxide anhydrous (98%), albumin, biotin labeled, bovine (b-BSA), albumin, from bovine serum (BSA), sulfuric acid (A.C.S reagent grade) were all obtained from Sigma-Aldrich. *o*-Phenylenediamine dihydrochloride (OPD) was purchased from Thermo-Scientific, which was stored at –20°C before use. Biotin-poly(ethylene glycol) amine (b-PEG Amine) with average molecular weights of 550 Da, 2000 Da, 3400 Da, 5000 Da, and 10,000 Da were all purchased from Layson Bio, Inc. Sodium phosphate dibasic and monobasic anhydrous, boric acid and borax (sodium borate) were all obtained from Fisher Scientific. HTS 96 well flat-bottom plates were obtained from Sarstedt, Inc. Citric acid (analytical reagent grade) was purchased from Mallinckrodt Chemical Works. All reagents were used as received unless specially described. All aqueous solutions were prepared using deionized water (> 18.0 MΩ•cm resistivity at 25 °C) obtained from Millipore Direct Q3 system.

2.2. Deposition of SIFs onto HTS Microplates

The deposition was achieved from our adopted standard procedure for the preparation of SIFs as described previously.(Abel et al., 2011) After the formation of SIFs in the reaction solution 300 μL of SIFs solution were then pipetted into the wells of the HTS microplates, which was later placed in a preheated oven at 60°C to warm up the reaction medium. To

accomplish the different extent of deposition (i.e., low, medium, and high loading), SIFs solutions in HTS microplates were kept in the preheated oven at 60°C at different time intervals [i.e., low (~1 minutes), medium (~3 minutes), and high (~5 minutes)] before removal of solution in the wells to achieve complete deposition of SIFs onto the HTS microplates.

2.3. Preparation of Solutions

All BEA compounds, b-BSA, BSA, and enzymes (i.e., HRP and AP) with their respective substrates (i.e., OPD and pNPP) were prepared as described in our previously published papers.(Abel and Aslan, 2014),(Abel and Aslan, 2012)

2.4. Colorimetric Assay

The colorimetric response of HRP or AP was determined based on the coupled reaction of HRP with OPD and AP with pNPP to produce a colored product. The colored product (30 μ L) were all taken from the SIFs-deposited HTS microplates after 2 minutes of incubation and placed in a separate HTS microplate containing their respective stopping reagents [200 μ L of 2.0 M of sulfuric acid] to stop the conversion of OPD, and [200 μ L of 3.0 M of sodium hydroxide] to stop the conversion of pNPP, followed by measuring absorption spectrum of the initial solutions (OPD, and pNPP), final product (colored product) and control solution. Control experiment, where SIFs were omitted from the surface was used to measure the extent of enzyme activity without the presence of SIFs.

2.5. Enzyme Adsorption on BEA-modified SIFs (High Loading)

A solution of 1 mg/mL (Absorbance at 280 nm ~ 1.0) of streptavidin-HRP and streptavidin-AP was prepared in phosphate buffer (pH = 7). These solutions were separately added to the all BEA-modified SIFs on HTS microplates and blank HTS microplates and allowed to incubate for at least 30 minutes at room temperature. The enzyme solution was removed from the HTS microplates and placed in a quartz cuvette, where the optical absorption spectrum (240 – 340 nm) was measured. The extent of protein characteristic absorption peak ~280 nm allowed us to semi-qualitatively compare the extent of enzymes adsorbed on to the surface. Subsequently, HTS microplates with enzymes immobilized on to SIFs were then washed using phosphate buffer to remove the unbound materials for further use.

2.6. Instruments

All colorimetric measurements were carried out using Cary 50 Bio UV-Spectrophotometer obtained from Varian, Inc. Digital images of SIFs-deposited HTS wells was taken by a 5 MP digital camera.

3. Results and Discussion

3.1. Characterization of SIFs

Figure 1 shows the real color photographs of SIFs-deposited HTS microplates, which visually confirms the presence of SIFs and the change in color as the extent of SIFs is increased from low to high loading on the HTS microplates. In order to optically

characterize the hybrid platform, we employed UV-Vis spectroscopy owing to the unique optical properties of the plasmonic nanoparticles. Figure 1 also shows the absorption spectra and the SPR peaks of SIFs deposited onto HTS microplates. As the extent of loading of SIFs is increased the SPR peak at 460 nm becomes prominent, which is accompanied by the broadening of the spectra >500 nm. These observations indicate an increased coupling of surface plasmons as the distance between individual silver islands are decreased. We note that the labeling of SIFs as low, medium and high loading is based on the value of the SPR peak at 460 nm, as indicated earlier in the text. We note that due to the physical challenges presented by the shape of HTS microplates microscopic (atomic force microscopy or scanning electron microscopy) analysis of SIFs was not carried out. In our previous work carried out on glass substrates,(Abel et al., 2011) the size of the SIFs were 50–90 nm for low, medium and high loading, and we expect the size of SIFs on HTS microplates to be similar since the same technique was employed.

3.2. Colorimetric response on SIFs

Figure 2 shows the summary of the results obtained for the absorption spectrum of the colored product obtained after the reaction of HRP and AP with their relevant substrates using BEA linker with average molecular weight: 3,400 Da and b-BSA on SIFs-deposited and blank HTS microplates (control sample). Figure 2A (HRP/BEA) and 2B (AP/BEA) show that as the loading of SIFs on HTS microplates is increased the absorption peak (for HRP: 495 nm, for AP: 405 nm) of the colored product also increases up to ~7-fold for both HRP and AP as compared to our control sample. The largest increase (~7-fold) in the colorimetric response for HRP and AP was observed for SIFs with high loading. We hypothesize that the use of BEA, which contains PEG spacer arm, was responsible for the enhanced colorimetric response based on the previous observations that BEA was shown to improve the conformation of antibodies to efficiently bind to antigens, which resulted in improved detection signals when employed in enzyme-linked immunosorbent assay (ELISA).(Wen et al., 2009) We note that BEA compound can be used in sandwich immunoassays to increase the loading of streptavidin or avidin-modified enzyme to increase the signal output of the assay. In this juncture, the use of SIFs and BEA offer an alternative under layer for the attachment of the antibodies and proteins and for improved conformation for enzymes for enhanced enzymatic reactions, respectively in bioassays carried out in HTS microplates.

In order to investigate the potential use of SIFs-modified HTS microplates for direct immunoassays, we employed b-BSA as the model target protein/antigen of interest. The absorption spectrum of the colored product obtained after the reaction of HRP and AP with their relevant substrates using b-BSA as the linker is shown in Figure 2C (HRP/b-BSA) and 2D (AP/b-BSA), respectively. Similar to the results obtained for BEA, as the loading of SIFs were increased the absorption peak for the colored products was increased up to ~6-fold for HRP and up to ~3-fold AP as compared to our control sample. In addition, the largest increase (~6-fold) in the colorimetric response for HRP and AP was observed for SIFs with loading. We note that b-BSA forms a monolayer on SIFs and HRP and AP are immobilized to the surface via biotin-streptavidin interactions. Since HRP and AP are less flexible on b-

BSA as compared to BEA, the efficiency of enzymatic conversion for HRP and AP on b-BSA was observed to be lower than when these enzymes were immobilized on BEA.

The comparison of the results obtained for BEA and b-BSA and the control experiments, i.e., the absorption peak values for colored products for HRP and AP was plotted and shown in Figure 3. The colorimetric response of HRP increases as the loading of SIFs increases from low to high as compared to control sample (Figure 2A). Similar results were also revealed using AP (Figure 2B). Figure 2A also shows that the increase in colorimetric response for HRP with b-BSA and BEA are comparable to each other. However, the use of BEA resulted in a larger increase in colorimetric response than b-BSA for all SIFs, as described earlier.

Based on the observation that use of SIFs with high loading in conjunction with BEA results in the largest increase in colorimetric response in our work, we investigated the effect of variation of chain length (spacer arm) of BEA on the colorimetric response by employing different molecular weight of BEA from 550 to 10,000-Da. We note that the spacer arm in BEA places the enzyme within a few nanometers away from the silver surface allowing for the effective interaction between the enzyme and substrate. The results obtained can be seen in Figure 3, which shows the absorption spectrum of the colored product using AP immobilized onto SIFs (high loading) deposited HTS microplates. We discovered that the use of BEA-5,000 Da yielded the highest colorimetric response as compared to other BEA linkers. We attribute the observation of largest colorimetric response to the nature of BEA (for example, BEA-5,000 Da places the enzyme ca. 10 nm from the surface (Heyes et al., 2004) and offers the most efficient conformation for enzymes to convert substrates in to colored products).

Optical spectroscopy was also employed to investigate whether there is a change in the extent of HRP and AP coverage on all surfaces with different BEA linkers. Figure 4 shows that the extent of HRP and AP coverage (indicated by absorption peak at 280 nm, characteristic peak for proteins) was similar on all surfaces, which implies that the increase in colorimetric response cannot be attributed to the increase in the extent of enzyme on the surface. Similar results were also observed for HRP and AP with the control sample (labeled as without BEA and SIFs in Figure 4). These findings were in agreement with the results presented in our previous paper in which a similar study was performed on planar glass substrates. (Abel and Aslan, 2014) These results imply that biological materials such as enzymes can be used with plasmonic nanoparticles for the improvement of their biological functions. In addition, the use of SIFs-modified HTS microplates as a solid platform will afford for the assessment of up to 96 samples at the same time with increased colorimetric signal. Subsequently, the use of SIFs can improve the lower detection limit of bioassays carried out in HTS microplates. Future applications of this new enzyme-based hybrid system will include design of a multiplexed assay system for detection of environmental pollutants and infectious agents and these findings will be reported in due course.

4. Conclusions

In summary, we have demonstrated that the use of HTS microplates with SIFs for the design of an enzyme-based hybrid platform that was capable of enhancing the colorimetric response of enzymes used in biological or environmental sensing. SIFs deposited at different extents of loading (i.e., low, medium, and high) within the HTS microplates were employed as our plasmonic nanoparticles in the construction of the hybrid system. To effectively immobilize the enzymes onto the plasmonic platform; b-BSA and BEA was used, which act as a linker for the immobilization of HRP or AP on the hybrid platform via biotin-avidin interactions. We observe from our results that AP immobilized to SIFs (high loading) deposited HTS microplates using b-BSA or BEA revealed the enhanced enzymatic colorimetric response as compared to our control sample.

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Abbreviations

SIFs	Silver island films
b-BSA	biotinylated-bovine serum albumin
BEA	biotinylated poly(ethylene-glycol)amine
HRP	horseradish peroxidase
AP	alkaline phosphatase

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Highlights

- 1** High throughput screening microplates are modified with silver island films
- 2** Biotinylated polymer and a protein used as linkers
- 2** Colorimetric response for HRP can be increased 7-fold
- 4** Colorimetric response of AP can be increased 6-fold

Figure 1.
(A) Real color photographs of SIFs (i.e., low, medium, and high) deposited on a HTS microplate. (B) Absorption spectrum of SIFs.

Figure 2.

Absorption spectrum of the colored product formed as a result of conversion of (A) OPD by HRP and (B) pNPP by AP immobilized by BEA-3,400 Da and (C) OPD by HRP and (D) pNPP by AP immobilized by b-BSA onto SIFs-deposited HTS microplates (low, medium, and high loading) and control sample (w/o SIFs).

Figure 3. Absorption peak values for the colored product formed as a result of conversion of A) OPD by HRP and (B) pNPP by AP. (C) Absorption spectrum of pNPP on SIFs-deposited HTS microplates using different molecular weight of BEA.

Figure 4.
Absorption spectrum of solution of (A) HRP and (B) AP after their incubation on SIFs-deposited HTS microplates.

Scheme 1.

Schematic depiction of Immobilization of enzymes onto SIFs-deposited HTS microplates using b-BSA or BEA. SIFs were omitted in the control samples.