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Enhancement of Colorimetric Response of Enzymatic Reactions by Thermally Evaporated Plasmonic Thin Films: Application to Glial Fibrillary Acidic Protein

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Abstract

We report the enhancement of the colorimetric response of horseradish peroxidase (HRP) and alkaline phosphatase (AP) in bioassays by thermally evaporated silver, gold, copper and nickel thin films. In this regard, a model bioassay based on biotin-avidin interactions was employed. Biotin groups and enzymes were introduced to all surfaces using a biotinylated linker molecule and avidin, respectively. The colorimetric response of HRP in the model bioassay carried out on the plasmonic thin films were up to 4.4-fold larger as compared to control samples (i.e., no plasmonic thin films), where the largest enhancement of colorimetric response was observed on silver thin films. The colorimetric response of AP on plasmonic thin films was found to be similar to those observed on control samples, which was attributed to the loss of enzymes from the surface during the bioassay steps. The extent of enzymes immobilized on to plasmonic thin films was found to affect the colorimetric response of the model bioassay. These findings allowed us to demonstrate the use of silver thin films for the detection of glial fibrillary acidic protein (GFAP), where the colorimetric response of the standard bioassays for GFAP was enhanced up to 67% as compared to bioassays on glass slides.

Keywords

Plasmonic thin films; silver thin films; gold thin films; copper thin films; nickel thin films; horse radish peroxidase; alkaline phosphatase; biotin-poly(ethylene-glycol)amine; glial fibrillary acidic protein; amplification of colorimetric response

Introduction

Enzymes are employed for the detection of target analytes via electrochemical,¹ fluorometric,² and colorimetric³ response, which is generated due to the catalytic reactions between the enzymes and their respective substrates. Colorimetric response is employed in enzyme-based bioassays more often than other types of response due to its simplicity, practicality and relatively low cost. However, the generated colorimetric response is limited by the dynamic range of the absorbance measurements (0 to 1) and requires further

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amplification steps to increase the detectability of the target analyte. To alleviate this problem, scientists employ a strategy whereby the extent of the enzymes is increased on the surface by physical means to generate additional colorimetric response.

Another strategy for the amplification of the colorimetric response is the incorporation of nanostructured materials into the enzyme-based bioassays.^{3–7} The success of this strategy can be attributed to the ability of nanostructured materials to manipulate the biological functions of enzymes due to their large specific surface area, high surface energy and electronic properties. For example, Zhou et. al. has demonstrated the amplification of colorimetric response of a bioassay for the detection of avian influenza virus (H9N2) using silver and gold nanoparticles, which is based on enzyme-induced metallization in solution.⁴ In the reported method, enzymes induced a silver deposition process on the surface of gold nanoparticles, which resulted in an increased change in the color of the detection solution.⁴ The detection limit for H9N2 was reported to be as low as 17.5 pg/mL.⁴ In addition, Lee and co-workers demonstrated a means of enhancing the colorimetric response of an immunoassay for the detection of interferon- γ using gold nanoislands and HRP.⁵ The authors discovered that the incorporation of gold nanoislands increased the sensitivity of the immunoassay, where a lower limit of detection 100-fold lower than the immunoassay without the gold nanoislands. The enhanced sensitivity was attributed to the increased number of molecules absorbed on the gold nanoislands, which led to an increase in the dielectric constant of local regions near the interface of the gold nanoislands.⁵

Despite the numerous advances in the development of novel strategies for colorimetric signal enhancement using nanostructured materials, there is still a need for improvement in terms of cost effectiveness, reduction in bioassay time, and simplification of the complex preparation methods. In this regard, the Aslan Research Group recently demonstrated a facile and inexpensive technique for the amplification of colorimetric response of commercially available enzymes such as HRP, AP, or β -galactosidase by plasmonic nanoparticles prepared via chemical synthesis route, i.e., silver island films (SIFs) and deposited on to silanized glass slides via chemisorption.^{6,7} The colorimetric response of HRP on SIFs was shown to be enhanced as the loading of SIFs on the glass surface was increased.⁶ Similar results were reported using AP on SIFs with an enhancement factor of 3-fold in comparison with surfaces without SIFs.⁷ However, chemically deposited SIFs were also shown to detach from the silanized glass surfaces during washing steps of the bioassays, which significantly limits the potential use of SIFs in real-life bioassays. In addition to silver and gold nanoparticles, there are reports on the use of other plasmonic nanoparticles, such as nickel, and copper in literature. Copper thin films have been applied as a catalyst sensing platform for glucose⁸ and employed along with carbon electrodes as a sensitive sensing system for the detection of amino acids.⁸ Previous studies have demonstrated that copper can also be used in bioassays.^{9, 10, 11} Nickel thin films have attracted attention due to their catalytic activity and electrical properties.^{12,13}

These studies afforded us to further investigate whether nanostructured materials deposited via thermal evaporation or sputtering can be used for the enhancement of colorimetric response of the enzyme-based bioassays. In this regard, we employed thermally evaporated 1 nm, 5 nm, and 10 nm silver, gold, copper, and nickel thin films as our plasmonic thin films

in conjunction with HRP and AP, the two most-widely used enzymes in bioassays. It is important to note the thermal evaporation and sputtering methods offer facile and homogeneous deposition of plasmonic thin films on to glass and polymeric surfaces, which is critical in their applicability in real-life bioassays. To assess the ability of plasmonic thin films to enhance the colorimetric response in bioassays, we initially employed a model bioassay based on biotin-avidin interactions. The immobilization of avidin-labeled HRP or AP onto plasmonic thin films was carried out using a biotinylated linker molecule (BEA with an average molecular weight of 3400 Da). We observed a significant increase in HRP and AP colorimetric response as the thickness of the plasmonic thin films was reduced from 10 to 1 nm. HRP colorimetric response was enhanced by 4.4-fold on silver thin films (1 nm thick), 2.50-fold on gold thin films (1 nm thick), 1.97-fold on copper thin films (1 nm thick), and 2.19-fold on nickel thin films (1 nm thick), as compared to the control sample. Additionally, AP colorimetric response was also enhanced by 1.13-fold on silver thin films (1 nm thick) and was reduced on gold, copper and nickel thin films.

Based on these findings, silver thin films (1 nm thick) were subsequently employed for the detection of GFAP, a useful diagnostic biomarker for various brain injuries¹⁴ and neurodegenerative disorders,^{15,16} using commercially available standard bioassays. In order to investigate whether the colorimetric response of the standard bioassay for GFAP can be further enhanced, the BEA linker molecule used in the model bioassays in this study was also employed (that is, the standard bioassay was modified by attaching BEA linker molecule to the secondary monoclonal anti-GFAP antibody in the standard bioassays). To confirm the effect of silver thin films on the enzymatic colorimetric response produced by the standard bioassays, a control surface (no silver thin films) was used. We observed that the enzymatic colorimetric response of a standard bioassay for GFAP can be enhanced up to 67% and the lower detection limit of 10 ng/mL for GFAP can be obtained by using silver thin films. These observations provide preliminary data for future design and construction of simpler and more sensitive biochemical sensors and point-of-care diagnostic systems based on plasmonics.

Materials and Methods

Materials

Hydrogen peroxide (30 wt. % solution in water), 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%), sulfuric acid (A.C.S reagent grade), *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), protein A from *Staphylococcus aureus*, phosphate buffered saline, potassium chloride (KCl), potassium phosphate monobasic (98%), Triton™ X-100 solution, sodium phosphate dibasic heptahydrate (Na₂HPO₄·7H₂O) (A.C.S reagent grade), and silane-prep glass slides were all obtained from Sigma-Aldrich. *O*-Phenylenediamine dihydrochloride (OPD) was purchased from Thermo-Scientific, which was immediately stored at −20°C before use.

Biotin-poly(ethylene glycol)amine (BEA) 3400-Da was brought from Laysan Bio, Inc. Human glial fibrillary acidic protein (GFAP) was procured from Abcam® (CA, USA). Monoclonal mouse anti-human glial fibrillary acidic protein, Clone 6F2, was obtained from Dako, North America, Inc. Peroxidase-labelled antibody to mouse IgG, human serum adsorbed, was purchased from KPL, Inc. PD-10 desalting columns for removal of low-molecular weight compounds was obtained from GE Healthcare Bio-Science Corp. Sodium phosphate dibasic and monobasic anhydrous, boric acid and borax (sodium borate) were all obtained from Fisher Scientific. Silver, gold, copper, and nickel targets (57 mm diameter × 0.1 mm thick), 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 flat-bottom plates were obtained from Sarstedt, Inc. Glass slides (micro slides, thickness: 0.96 to 1.06 mm) used in this work was obtained from Corning Inc. Citric acid (analytical reagent grade) was purchased from Mallinckrodt Chemical Works, and ethyl alcohol (200 proof) were bought from Pharmco. Triethoxysilylbutyraldehyde was bought from Gelest, Inc. All reagents were used as received unless specially described. All aqueous solutions were prepared using deionized water ($> 18.0 \text{ M}\Omega\cdot\text{cm}$ resistivity at 25°C) obtained from Millipore Direct Q3 system except stated otherwise.

Methods

(1) Deposition of Silver, Gold, Copper, and Nickel Thin Films onto Glass

Slides—Plasmonic thin films were deposited onto glass slides using silver, gold, copper, and nickel targets and a sputter coater (EMS 150R, equipped with film thickness monitor) obtained from Electron Microscopy Science (PA, USA). The thickness of the plasmonic thin films was adjusted to 1 nm, 5 nm, and 10 nm using an inbuilt film thickness monitor. [Note: All plasmonic thin films were kept in a desiccator before use to minimize surface oxidation of the metal surface].

(2) Characterization of Plasmonic Thin Films using Atomic Force Microscopy

(AFM)—Plasmonic thin films (1 nm, 5 nm and 10 nm thick) were deposited onto silanized glass slides by a sputter coater. To confirm the deposition of plasmonic thin films, we monitored the characteristic surface plasmon resonance peak (SPR) of the plasmonic thin films by measuring their UV-visible absorption spectrum. In addition, the deposition of 1, 5, and 10 nm thick gold thin films on silane-prep glass substrate was also confirmed by a table-top AFM. The results were provided as 2D image, line scan and height profile. The gold thin films were observed to retain their planar character with an average height of (1.26 ± 0.31 nm, 4.01 ± 1.27 , and 9.02 ± 1.34 nm) respectively, which is agreeable to the known size of the nanoparticles (i.e., 1, 5, and 10 nm). The nanoscale morphology of the gold films indicates the formation of a monolayer.

(3) Surface Modification of Plasmonic Thin Films with BEA—BEA was prepared and attached onto silver, gold, copper, and nickel thin films by adopting the procedure from our previous work,¹⁷ schematic depiction is shown in Scheme 1.

(4) Surface Modification of Glass Substrates with Organosilanes (Control

Surface)—The procedure for the surface modification of glass slides using an organosilane

has being previously described.¹⁸ The modified glass slides were used as our control surface for the immobilization of BEA. [Note: Plasmonic thin films were omitted on the control surface].

(5) Preparation of OPD and pNPP solutions—The preparation of OPD solution was carried out as described previously.⁶ The corresponding wavelength of the color product (2, 3-diaminophenazine) obtained after interaction of OPD with HRP-avidin was at 495 nm in all experiment. In addition, pNPP solution was used directly from the alkaline phosphate yellow (pNPP) liquid substrate obtained from the manufacturer without any further dilution. The wavelength of the color product (*p*-nitrophenol) obtained after the interaction of pNPP with AP-avidin was at 405 nm.

(6) Colorimetric Bioassay for the Evaluation of Plasmonic Thin Films for Enhanced Colorimetric Response—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 color product (Scheme 1). The substrate OPD was prepared as described⁶ whereas pNPP was used as received from the manufacturer. The colored products (30 μ L) were all taken from the plasmonic thin films after 2 minutes of incubation and placed in high-throughput screening microplates containing their respective stopping reagents (i.e., 2.0 M of sulfuric acid (200 μ L) to stop the conversion of OPD, and 3.0 M of sodium hydroxide (200 μ L) to stop the conversion of pNPP). Subsequently, we measured the absorption spectrum of the initial solutions OPD, pNPP, final colored product and control solution. Control experiment, where the plasmonic thin films were omitted from the surface was used to measure the extent of enzymatic colorimetric signal enhancement without the presence of plasmonic thin films.

(7) Preparation of Standard Bioassays for GFAP on Silver Thin Films (1 nm thick)—The standard bioassay was prepared as previously described¹⁹ and its schematic depiction is shown in Scheme 2. The standard bioassay is based on the deposition of protein A (10 μ g/mL in a sample buffer [PBS (137 mM of NaCl, 2.7 mM of KCl, 1.4 mM of KH_2PO_4 , and 8.0 mM of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ at pH 7.4) containing 0.5% Triton X-100]) to 1 nm thick silver thin films which supports the binding of monoclonal mouse anti-GFAP antibody. The protein A was incubated onto the silver surface for 24 hours at 4°C which was then washed with PBS solution to remove the unbound proteins. Furthermore, monoclonal anti-GFAP antibody (dilution 1:500 in PBS containing 0.5% Triton X-100) was then incubated onto the modified silver surface for 2 hours at 4°C, before washing with PBS solution to remove unbound antibody from the silver surface. The monoclonal anti-GFAP antibody serves as the capture reagent for binding GFAP at different concentrations (1–100 ng/mL). Subsequently, GFAP bound to the capture antibody is then bound by a secondary monoclonal mouse anti-GFAP antibody which was incubated for 2 hours at 4°C and later washed with PBS solution to remove unbound materials. In addition, the secondary anti-GFAP antibody is then bound by HRP-labeled polyclonal antibody to mouse IgG (dilution 1:3000 in PBS solution containing 0.5% Triton X-100) which is directed toward it, but not the capture antibody. In the control experiments, several components were omitted from the bioassay: (i) no silver thin films (i.e., blank glass surface), (ii) no GFAP (control 1), and (iii)

no silver thin films and no GFAP (control 2). The quantification of GFAP was achieved via colorimetric measurements of the bound HRP on the different surfaces.

(8) Preparation of Modified Standard Bioassay for GFAP on Silver Thin Films using BEA-Modified Secondary Antibody—This procedure was achieved in a two-step process: (i) modification of the secondary monoclonal anti-GFAP antibody with BEA, and (ii) binding of the BEA-modified secondary anti-GFAP antibody to GFAP immobilized on 1 nm thick silver thin films as shown in Scheme 3. In Scheme 3A, secondary monoclonal mouse anti-GFAP antibody was reacted with 10 μ L of EDC (4 mg/mL) in PBS solution for 2 minutes. In this process, EDC reacts with the carboxyl group on the antibody and forms an intermediate (*o*-acylisourea ester), which is known to be unstable in aqueous solution.²⁰ The intermediate is then reacted with 5 μ L of NHS (22 mg/mL) in PBS solution for 2 minutes to form a semi-stable amine reactive NHS ester that is stable for few hours.²⁰ Subsequently, BEA (3400 Da) was then added into the solution to covalently crosslink to the secondary anti-GFAP antibody. The final solution was then passed through a PD-10 desalting column used for the molecular separation of proteins on the basis of their differences in size. The larger molecules (BEA-modified antibody) were excluded from the column and are eluted first, whereas the smaller molecules were retained in the column. Furthermore, BEA-modified secondary anti-GFAP antibody is then incubated on to GFAP immobilized to 1 nm thick silver thin films for 2 hours at 4°C as shown in Scheme 3B, before washing with PBS solution to remove unbound antibody. In addition, BEA-modified secondary antibody is then bound by streptavidin conjugated HRP via biotin-avidin interaction. In the control experiments, several components were omitted from the bioassay: (i) silver thin films (i.e., when glass surface was used), (ii) GFAP (control 3), and (iii) silver thin films and GFAP (control 4). In order to quantify the GFAP on the bioassay, colorimetric measurements of the bound HRP was retrieved after the reaction with OPD.

Instrumentation

In this work, all absorption measurements were carried out using Cary 50 Bio UV-Spectrophotometer obtained from Varian, Inc. AFM images of gold thin films were taken using a table-top atomic force microscope obtained from AFM workshop Inc. In addition, digital images of silver, gold, copper, nickel-deposited silane-prep glass slides before and after use was taken by a 5 MP digital camera.

Results and Discussion

Figure 1 shows the absorption spectrum and the corresponding SPR peaks of 1 nm, 5 nm, and 10 nm thick silver, gold, copper, and nickel thin films. Figure 1A shows the absorption spectrum and real-color photographs of 1 nm, 5 nm, and 10 nm thick silver thin films. Silver thin films displayed different colors for 1 nm (light gray), 5 nm (light blue gray), and 10 nm (dark blue gray) thick films. The absorbance spectrum of 1 nm thick silver thin films exhibit a dominant SPR peak around 430 nm, which implies that silver was deposited as a nanoparticle film. However, the absorbance spectrum for 5 nm and 10 nm silver thin films broadened at wavelengths >450 nm, which indicates that the distance between the silver

nanostructures decrease and a semi-continuous thin film is formed on the surface (that is the surface becomes reflective).

Figure 1B shows the absorption spectrum and real-color photographs of 1 nm, 5 nm, and 10 nm thick gold thin films. While 1 nm thick gold thin films appeared to have the characteristic pinkish color for colloidal gold nanoparticles, the color of the 5 nm and 10 nm thick gold thin films light green to dark green, respectively. The SPR peak of 1 nm thick gold thin films occurred at 550 nm. Similar to the silver thin films, the increase in thickness of the gold thin films resulted in the broadening of the absorbance spectrum at wavelengths > 600 nm. These observations are consistent with the results obtained from other studies on the optical properties of gold thin films on glass substrates.²¹ Figure 1C and 1D show that copper and nickel thin films did not display a strong SPR peak in the 300–700 nm region of the electromagnetic spectrum. As shown by other research groups, colloidal copper of 30 nm in diameter in solution are expected to display a SPR peak around 600 nm.²² However, the absorption spectrum of copper thin films (CuO) is different than the colloidal copper due to the lower band gap of the thin films than the colloidal copper.²³ A weak SPR peak for copper thin films occurs at 350 nm and the absorption value for the SPR peak increases as the thickness of the copper thin films increases to 10 nm, which is accompanied by the broadening of the absorption spectrum at longer wavelengths. The broadening of the absorption spectrum can be attributed to the aggregation of the nanoparticles on the glass surface. Similar observations were also made for nickel thin films and the absorption spectrum presented in Figure 1D is consistent with the absorption spectrum for nickel thin films reported in literature.²⁴

Figure 2 shows the overall comparison of the absorption colorimetric response in a model bioassay for the target molecule (i.e., biotin) at the absorption peak value at 495 nm of HRP and AP on all plasmonic thin films and control samples used in this study. It is important to note that these values are obtained from the absorption spectrum of the color product (2,3-diaminophenazine) collected after the hydrolysis of OPD by HRP immobilized on to 1 nm, 5 nm, 10 nm thick silver, gold, copper, and nickel thin films and the control surfaces (Figure S4, Supporting Information). As shown in Figure 2A, the absorption value of the colored product on the plasmonic thin films shows an increase as the film thickness is reduced to 1 nm, as compared to the control surface, where the plasmonic thin films were omitted from the surface. In addition, the colorimetric response of HRP on 10 nm thick silver, gold and nickel thin films were similar to those measured on the control sample. The enhancement factor for the colorimetric response as a result of enzymatic activity on plasmonic thin films was calculated according to the following formula: the net enzymatic response observed on plasmonic thin films divided by the net enzymatic response observed on the control samples, and are shown in Supporting Information (Table S1). The enhancement factors for the colorimetric response were ~ 4.4-fold for 1 nm thick silver thin films and ~2.5-fold for the other 1 nm thick plasmonic thin films. The largest enhancement of enzymatic activity for 5 nm thick plasmonic thin films was ~ 3-fold for silver thin films.

To assess the effect of extent of surface immobilized enzyme on the observed enhancement of enzymatic activity, we have measured the absorbance of enzymes immobilized on all surfaces at 280 nm and calculated the weight of enzyme per unit area.⁷ Table S1 shows that

the extent of HRP coverage on all 1 nm thick plasmonic thin films were slightly higher for silver and gold and were comparable for copper and nickel to the control sample; (i) silver (23 ± 1.8 ng/mm²), gold (23 ± 2.4 ng/mm²), copper (21 ± 1.2 ng/mm²), nickel (19 ± 6.4 ng/mm²), control sample (18 ± 2.0 ng/mm²), respectively. These results indicate that the extent of enzyme immobilized on to the plasmonic thin films has a slight effect on the observed enhanced enzymatic activity. Moreover, since the enzymes were placed within a few nanometers from the plasmonic thin films, the enhancement in colorimetric response obtained can be attributed to the electronic properties of the plasmonic thin films, where the plasmonic surface are thought to affect the electron transport processes involved in the enzymatic reactions. These suggestions were also made by other research groups, for example, Chen *et al.* have recently demonstrated a technique known as “Plasmon-Enhanced Enzyme-Linked Immunosorbent Bioassay”,^{25, 26,26} where an optically dense precipitate catalyzed by enzyme can be generated by metal surfaces.

In addition, it is thought that the detachment of enzymes from the surfaces during incubation and wash processes in the model bioassays can also affect the extent of colorimetric response. To confirm whether the observation of decreased colorimetric response can be attributed to loss of plasmonic thin films from the surfaces, the digital images of the plasmonic thin films were taken before and after the model bioassays were carried out (Supporting Information, Table S2). Since the plasmonic thin films change the color of the glass slides and can be visually detected, digital images provide a facile method for qualitative characterization of plasmonic thin films before and after the model bioassays are carried out. The digital images show that 5 nm and 10 nm silver thin films show a significant loss of color, which indicates the enzymes along with plasmonic thin films were lost during washing of the surface and contributed to the lower colorimetric signal as compared to 1 nm thick silver thin film that displayed no loss of color. The loss of color of the gold, copper and nickel thin films were less pronounced for all thicknesses, which implies that the loss of enzyme from the surface did not significantly contribute to the observed colorimetric response from these thin films.

To corroborate to the observations of lower colorimetric response on 5 nm and 10 nm thick plasmonic thin films, we further investigated the effect of the extent of surface immobilized enzyme on the enhancement of enzymatic activity for 5 nm and 10 nm thick plasmonic thin films (Supporting Information, Table S4–S5). The extent of HRP on 5 nm thick plasmonic thin films were determined to be as follows: (i) silver (16 ± 1.14 ng/mm²), gold (9 ± 3.0 ng/mm²), copper (12 ± 2.42 ng/mm²), nickel (11 ± 1.3 ng/mm²), and on 10 nm thick plasmonic thin films: (i) silver (3 ± 0.57 ng/mm²), gold (3 ± 0.79 ng/mm²), copper (4 ± 1.03 ng/mm²), nickel (3 ± 0.37 ng/mm²), respectively. These results imply that the extent of HRP on the 5 nm and 10 nm thick plasmonic thin films are significantly lower than that on glass slides, and were deemed to be the one of the reasons for the low enzymatic response observed for 5 nm and 10 nm thick silver, gold, copper and nickel thin films.

In addition to the use of HRP with plasmonic thin films, we investigated whether the variation in the thickness of plasmonic films can affect the colorimetric response of AP. In this regard, Figure 2B shows the overall comparison of the absorption values at 405 nm for silver, gold, copper, and nickel thin films, and control experiment. Figure 2B clearly shows a

slight enhancement colorimetric response of AP on 1 nm thick silver and gold thin films as compared to control sample. However, the colorimetric response for AP at 405 nm obtained from the control sample was similar to 1 nm thick copper and nickel thin films. (Figure S5 in Supporting Information shows the absorption spectrum obtained after the conversion of pNPP to the colored product (*p*-nitrophenol) by AP immobilized onto 1 nm, 5 nm, 10 nm thick silver, gold, copper, and nickel thin films and control samples). As shown in Figure 2B, the peak absorption value at 405 nm for the colored product produced by AP on plasmonic thin films is increased as the thickness of thin films is decreased. However, the absorption peak value at 405 nm on the control sample was observed to be comparable to the values observed for all plasmonic thin films. Detailed discussion related to the colorimetric response of AP on plasmonic thin films can be found in the section 2 of the supporting information.

Subsequent to the successful demonstration of enhanced enzymatic colorimetric response of HRP in a model bioassay with silver thin films, we investigated whether 1 nm thick silver thin films can also enhance enzymatic colorimetric response of commercially available bioassays for GFAP. In this regard, we employed two strategies: i) a standard bioassay for GFAP¹⁹ was prepared on silver thin films (Scheme 2) and ii) a modified standard bioassay for GFAP was carried out by using secondary monoclonal antibody modified with BEA (Scheme 3) on silver thin films. In control experiments, GFAP was omitted from the standard bioassays to assess the effect of non-specific detection of GFAP. In this regard, Figure 3 (and Figure S7 in Supporting Information) shows the results of the colorimetric response of the standard bioassay and the modified standard bioassay for GFAP for concentration range of 1–100 ng/mL on silver thin films and glass slides. Figure 3 shows that the absorption peak values for the colored product increases as the GFAP concentration increases on all bioassays. The colorimetric response for the concentration range of GFAP in the modified standard bioassay is significantly higher than those observed on the standard bioassay on silver thin films (Figure 3A). The background absorbance levels for HRP on all bioassays were determined from the control bioassays (control 1: with silver thin film and without GFAP and control 3: with BEA and without GFAP).

In addition, Figure 3B shows the absorption peak values for the standard bioassay and modified standard bioassay for GFAP carried out on glass slides (without silver thin films). The colorimetric response using the modified standard bioassay is significantly higher than those observed from the standard bioassay carried out on glass slides. This observation can be attributed to the use of BEA in the modified standard bioassay. To determine the background absorbance levels for HRP on all bioassays, several control bioassays were carried out: control 2: glass without silver and without GFAP, and control 4: glass-BEA without silver and without GFAP. The lower detection limit for GFAP was determined to be 10 ng/mL for both bioassays on silver thin films and was 5 ng/mL on glass slides. However, the dynamic range for the detection of GFAP using both standard bioassays was significantly improved on silver thin films as compared to bioassays carried out on glass slides. The observation of the largest colorimetric response on modified standard bioassay on silver thin films in this study can be attributed to the electronic properties of silver thin films (reasons stated earlier in the text) and to the linker molecule BEA, which is known to

improve the conformation of antibodies bind to antigens which has resulted in increased detection signals when employed in immunoassays.²⁷

In order to investigate the effect of the extent of immobilized HRP on the colorimetric response in bioassays for GFAP, the extent of HRP on 1 nm thick silver thin films were determined by measuring absorbance at 280 nm are shown in Table S6 (Supporting Information). These results reveal that the extent of HRP for all samples was increased as the extent of GFAP was increased on all bioassays. Moreover, the extent of HRP on silver thin films in modified standard bioassay was slightly higher as compared to silver thin films used in standard bioassays, which can be attributed to the availability of increased binding sites for HRP-labeled avidin in the modified standard bioassays. The observations of increased colorimetric response in the modified standard bioassays (Figure 3A), where BEA-modified secondary monoclonal antibody and HRP-labeled avidin was employed, can be partially attribute to the increased extent of HRP in these bioassays.

We also investigated whether the detachment of silver thin films, attachment of colored product to silver thin films and/or enzymes from the surface during incubation and multiple washing steps also affect the colorimetric response in GFAP bioassays. In this regard, the absorption spectrum of the silver thin films before and after the standard bioassay (Figure 4A) and the modified standard bioassay (Figure 4B) was measured and shown in Figure 4. Figure 4A shows that the SPR peak of silver thin films at 430 nm (line 1: before the standard bioassays) is decreased and exhibited a red and blue shift as the concentration of GFAP increased (lines 2–7: after the standard bioassays). The SPR peak of silver thin films is decreased and exhibited a blue shift accompanied by the narrowing of the absorption spectrum at shorter wavelengths <430 nm after the modified standard bioassay was carried out (Figure 4B). The blue shift in the SPR peak of silver thin films implies that the distance between the silver nanostructures increases due to the loss of silver thin films from the surface. The red shift in the SPR peak and the broadening of the spectrum of silver thin films can be attributed to the change in the dielectric constant of the silver thin films due to the attachment of the colored product, which has a peak at 495 nm. It is important to note that the decrease in the SPR peak is a constant ~33 % (from 0.09 to 0.06) for all concentrations of GFAP implies that there is a change in the dielectric constant of the silver thin films and/or silver thin films are released in a similar fashion from the surface after the completion of the bioassays. However, since the silver thin films contain concentration-dependent amount of GFAP and avidin-labeled HRP, the potential presence of silver thin films with GFAP/avidin-labeled HRP in the solution containing the colored product can influence the outcomes of the colorimetric response of the bioassays.

In order to experimentally demonstrate that in fact proteins are present on the solution containing the colored product, the absorption spectrum of the colored product was also measured to cover the absorbance wavelength range of 240–600 nm (Figure S8, Supporting Information). Figure S8 shows that the absorbance value at 280 nm is increasing as the concentration of GFAP is increased. This observation directly implies that proteins (i.e., protein A, antibodies, GFAP and/or avidin-labeled HRP) are present in the solution along with the colored product during the completion of the last step (conversion of OPD to the colored product) of the bioassays, since proteins absorbs light at 280 nm. The extent of

proteins was found to be more significant at GFAP concentrations >25 ng/mL. This can also imply that one or more proteins in the bioassays are detached from the surface with silver thin films after the conversion of the substrate to the colored product. Since these proteins have similar sizes, we did not attempt to identify the proteins present in the solution containing the colored product. Subsequently, it can be concluded that the colorimetric response measured in GFAP standard bioassays (Figure 4) for GFAP concentrations >25 ng/mL is slightly affected by the detachment of the silver thin films, while these bioassays displayed a concentration-dependent colorimetric response.

Conclusions

In this work, we have demonstrated the use of 1 nm, 5 nm, and 10 nm thick silver, gold, copper, and nickel thin films for the enhancement of colorimetric response of HRP and AP using a model and real-life bioassays. The fabrication of plasmonic thin films onto silanized glass slides was done using a sputter coater, which is a low cost and rapid means of producing thin films on solid substrates. The potential for plasmonic thin films to enhance the colorimetric response was first evaluated using a model biotin-avidin bioassay, where a biotinylated linker molecule was employed as the molecule. The colorimetric response of HRP immobilized on 1 nm thick silver, gold, copper, and nickel thin films showed enhanced colorimetric response (largest enhancement was on 1 nm silver thin films) as compared to film thickness of 5 nm and 10 nm in the model bioassays. Similar results were also revealed from the colorimetric response of AP immobilized to plasmonic thin films in the model bioassays. These findings suggest that enhanced enzymatic colorimetric response for HRP could be achieved by incorporating semi-continuous films of silver, gold, copper, and nickel thin films into current bioassay platforms, such as glass slides. However, AP was revealed to be enhanced by semi-continuous films of silver and gold, respectively. Subsequently, we were able to successfully demonstrate that the incorporation of 1 nm silver thin films and the biotinylated linker molecule in to standard bioassays for a biologically relevant diagnostic protein, i.e., GFAP. We observed up to 67% increase in the colorimetric response from the standard bioassays for GFAP. These results imply that colorimetric response of biological bioassays can be improved with the incorporation of plasmonic thin films. Subsequently, the use of BEA-modified monoclonal secondary antibody can further increase the colorimetric response of bioassays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Absorption spectrum of (A) silver, (B) gold, (C) copper, and (D) nickel thin films (1 nm, 5 nm, and 10 nm thick). Insert-real-color photographs of the plasmonic thin films.

Figure 2. Colorimetric response at the peak value of (A) HRP and (B) AP on all plasmonic thin films and control surface used in the model bioassays.

Figure 3. Colorimetric response of HRP at 495 nm in standard bioassays and modified standard bioassays for GFAP at different concentrations (1–100 ng/mL) on (A) silver thin films (1 nm thick) and (B) glass slides.

Absorbance of Silver Thin Films Before and After Standard Bioassay and Modified Standard Bioassay for GFAP

Figure 4.

Absorption spectrum of silver thin films (1 nm thick) before and after **(A)** standard bioassays and **(B)** modified standard bioassays for GFAP at different concentrations (1–100 ng/mL).

Scheme 1.

Schematic depiction of enzymes deposited onto silver, gold, copper, nickel thin films using biotin-avidin interactions. Plasmonic thin films were omitted in the control bioassays.

Scheme 2.

Schematic depiction of the standard bioassays for GFAP on silver thin films (1 nm thick): the standard bioassays are based on the deposition of protein A onto silver thin films, which supports the binding of monoclonal mouse anti-GFAP antibody. The antibody serves as the capture reagent for binding GFAP at different concentrations (1–100 ng/mL). Subsequently, GFAP bound to the capture antibody is then bound by a secondary antibody. This secondary antibody is then bound by an enzyme-linked antibody (HRP-labeled polyclonal antibody to mouse IgG). Quantification of GFAP is achieved via the measurement of the colorimetric response of HRP. The control experiments were carried out on glass slides without plasmonic thin films, where GFAP (control 1), silver thin films and GFAP (control 2) were omitted from the standard bioassays.

Scheme 3.

Schematic depiction of the modified standard bioassay for GFAP on silver thin films (1 nm thick) using biotin-poly (ethylene-glycol)-amine (BEA) modified secondary antibody: This procedure was achieved in a two-step process; (A) EDC and NHS reactions were employed to crosslink BEA to secondary monoclonal mouse anti-GFAP antibodies. (B) The modified standard bioassay which is based on the deposition of protein A onto silver thin films, which supports the binding of monoclonal mouse anti-GFAP antibody. The antibody serves as the capture reagent for binding GFAP at different concentrations (1–100 ng/mL). Subsequently, GFAP bound to the capture antibody is then bound by BEA modified secondary antibody in (A). This BEA modified secondary antibody is then bound by streptavidin conjugated enzyme via biotin-avidin interaction. Quantification of GFAP is achieved via the measurement of the colorimetric response of HRP. The same control experiments used in standard bioassays are also used in the modified standard bioassays.