

# Plasmon-Enhanced Enzymatic Reactions 2: Optimization of Enzyme Activity by Surface Modification of Silver Island Films with Biotin-Poly (Ethylene-glycol)-Amine

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## Abstract

Surface modification of silver island films (SIFs) was carried out with Biotin-Poly (Ethylene-glycol)-Amine (BEA), which acts as a cross-linker between the silver surface and horse radish peroxidase (HRP) enzyme for optimum plasmon-enhanced enzymatic activity. SIFs-deposited blank glass slides and SIFs-deposited 3-Aminopropyltriethoxysilane (APTES)-coated glass slides were used as our plasmonic surfaces. In this regard, three different extent of loading of SIFs were also prepared (low, medium and high) on APTES-coated glass slides. Streptavidin-linked HRP enzyme was attached to SIFs-deposited blank glass slides and SIFs-deposited APTES-coated glass slides through the well-known biotin-streptavidin interactions. The characterization of these surfaces was done using optical absorption spectroscopy. The loading of SIFs on glass slides was observed to have significant effect on the efficiency of plasmon-enhanced enzymatic activity, where an enhancement of 200% in the enzymatic activity was observed when compared to our previously used strategies for enzyme immobilization in our preceding work [1]. In addition, SIFs-deposited on APTES-coated glass slides were found to be re-usable for plasmon-enhanced enzymatic reactions unlike SIFs deposited on to blank glass slides.

**Keywords:** Plasmon-enhanced enzymatic reactions, surface plasmons, surface plasmon resonance, silver island films, gold nanoparticles, horse radish peroxidase, enzymatic activity.

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## 1. Introduction

In the last two decades, several new plasmonic technologies that employ metal nanoparticles and planar thin films for the detection of biomolecular interactions have emerged: surface plasmon resonance analysis, surface enhanced Raman scattering and surface plasmon enhanced fluorescence spectroscopy [2, 3]. In addition, the combined use of enzymes with plasmonic structures was shown to enable us design new hybrid enzyme-nanoparticle systems for applications in bio- and nanotechnology. It was previously shown that plasmonic nanoparticles enhance the electro-catalytic response of a glucose oxidase system (glucose biosensor) [4] and the detection of various analytes of interest, such as hydrogen peroxide and uric acid [5], thiocholine [6], carcinoembryonic antigen [7] and DNA [8]. More recently, Aslan Research Group recently presented a detailed investigation of the dependence of enzymatic

activity on the nanoparticle-enzyme distance and nanoparticle loading on planar surfaces based on the new platform technology called plasmon-enhanced enzymatic reactions (PEER). In this regard, three different SIFs (low, medium and high loading) on APTES-coated glass slides and unsilvered (blank) APTES-coated glass slides (control samples) were used for the comparison of three different enzyme immobilization strategies for PEER. Three different strategies: 1) a biotin-avidin protein assay (strategy 1), 2) self-assembled monolayer of hexamethylenediamine (strategy 2) and 3) poly-L-lysine layer (strategy 3) were used to vary the distance of the enzyme from the silver surface. It was found that up to an 200% increase in enzymatic conversion of organic substrate by horse radish peroxidase (HRP) was observed from SIFs with high using strategy 1 compared to control samples, providing direct evidence that plasmon-

enhanced enzymatic activity is highly dependent on the enzyme-nanoparticle distance and the extent of loading of silver nanoparticles.

In this paper, we used a compound (BEA) that has terminal biotin groups, polymer chain and an amine terminal group for the surface modification of SIFs to optimize plasmon-enhanced enzymatic activity of HRP on SIFs. Since most biologically active compounds, such as enzymes are known to lose activity when linked directly to a solid hydrophobic surface, we hypothesized that the use of a hydrophilic molecule (such as BEA) that has a spacer arm poly-ethylene glycol may shield the enzyme from denaturation and improve bioactivity [9-11]. Poly-ethylene glycol is routinely used to attach immunogens to a solid surface, where one can observe a reduction in non-specific enzyme absorption and increased enzyme stability. These factors are seen to be critical in optimizing the specificity and sensitivity of bioanalytical assays [10, 11]. Poly-ethylene glycol has also been used as a spacer molecule for the improvement peptide bioactivity [11], and antigen detection in biosensor [12]. In this paper, the comparison between the use of SIFs-deposited blank glass slides and SIFs-deposited-APTES-coated glass slides for optimization of PEER is described. The plasmon-enhanced enzymatic activity was followed by the colorimetric measurement of the product produced as a result of enzymatic conversion of o-phenylenediamine (OPD) on silvered surfaces. In addition, the effect of loading of SIFs onto APTES-coated glass slides (low, medium and high) as an optimization parameter was carried out.

## Materials and Methods

**Materials.** Biotin-poly(ethylene-glycol)-amine (BEA) was obtained from Laysan Bio Inc. Sodium phosphate monobasic anhydrous was obtained from Fisher Scientific. Sodium hydroxide (99.99%), streptavidin-peroxidase from streptamycsavidinii (HRP-streptavidin), D-glucose, 3-Aminopropyltriethoxysilane (APTES) (99%), ammonium hydroxide (30%) and nitric acid (A.C.S reagent grade) were all obtained from Sigma-Aldrich. Culture well chambered cover glass (2 wells, 15 mm diameter, 2.0 mm deep) used for this work, were obtained from Electron Microscopy Sciences (Hatfield, PA). O-phenylenediaminedihydrochloride (OPD) was obtained from Thermo-Scientific. Silver nitrate (A.C.S reagent grade) was purchased from Spectrum Chemical Corporation. Citric acid (analytical reagent grade) was purchased from Mallinckrodt Chemical Works. Sulfuric acid (A.C.S reagent grade) was purchased from Pharmco product Inc. All solutions were prepared using deionized water obtained from Millipore Direct Q3 system.

**Methods. (1) Deposition of Silver Island Films (SIFs) on Glass Slides.** The glass slides were first cleaned in piranha solution (70% sulfuric acid, 30% hydrogen peroxide). The slides were rinsed with deionized water, then kept to dry at room temperature. The cleaned glass slides were later immersed in an ethanolic solution containing 2% (3-aminopropyltriethoxysilane) APTES

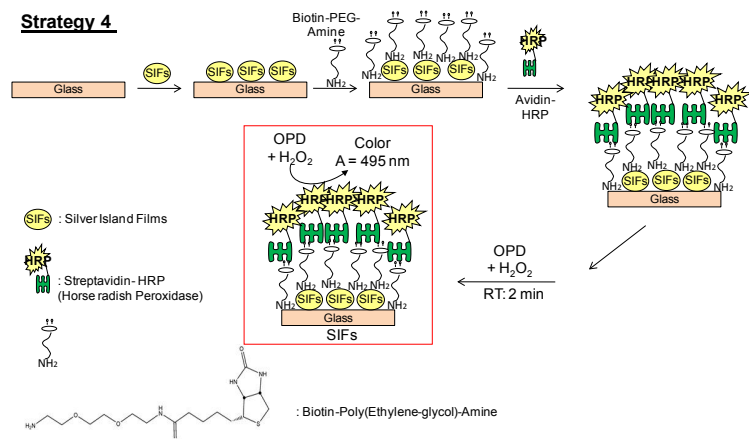
for 1 hour. The APTES-coated glass slides were rinsed with ethanol and allowed to dry at room temperature. The preparation and deposition of SIFs on to blank glass slides and to the APTES-coated glass slides at different loading (low, medium and high) was carried out as in the previously described procedure [1]. The characterization of the slides was done using UV-Visible spectrophotometer, where absorbance spectra of SIFs-deposited glass slide and SIFs-deposited APTES-coated glass slides were recorded, indicating the relative amount of silver nanoparticles to the surface of the glass slides.

**(2) Preparation of Biotin-Poly(Ethylene-glycol)-Amine assay on SIFs and SIFs-deposited APTES-coated glass slides.** BEA, which has a biotin group attached to an ethylene-glycol core and a terminal amine surface group, was used for surface modification of SIFs surfaces, as shown in Scheme 1. BEA was prepared at a concentration of 0.5 mM using sodium phosphate monobasic buffer solution at pH 7. Culture well chambered cover glass was used to cover the SIFs-deposited glass slide and SIFs-deposited APTES-coated glass slides before the addition of 200  $\mu$ L of BEA solution in the well chambers, the incubation was kept for 30 minutes. The slides were rinsed with deionized water to remove the unbounded BEA [Note: Incubation longer than 30 minutes will result in the rapid loss of SIFs on the surfaces of both glass slides]. HRP-streptavidin (~200  $\mu$ L) solution was subsequently added into the wells containing BEA-coated with SIFs-deposited glass slide and SIFs-deposited APTES-coated glass slides. The preparation of HRP-streptavidin was done as described previously [1]. The slides were then rinsed with deionized water to remove the unbounded HRP-streptavidin. HRP-streptavidin was used with BEA for the preparation of the monolayer by specific interaction with biotin group.

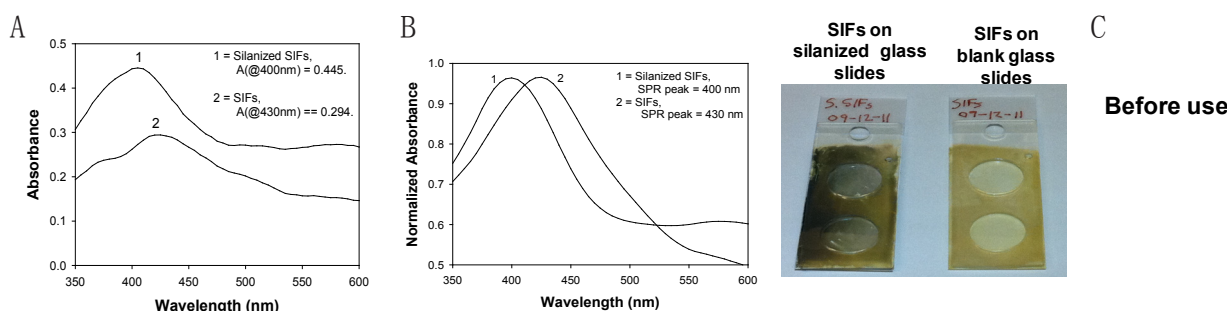
**(3) Preparation of o-phenylenediaminedihydrochloride (OPD) solution.** The preparation of OPD solution was carried out as described previously [1]. The wavelength of the product obtained after the interaction of OPD with HRP-streptavidin was at 495 nm for all experiments. **Instrumentation.** Cary 50 Bio UV-Visible spectrophotometer (Varian, Inc.) was used to obtain all absorption measurements. Real-color photographs of SIFs before and after use were taken by a 5 MP digital camera.

## Results and Discussion

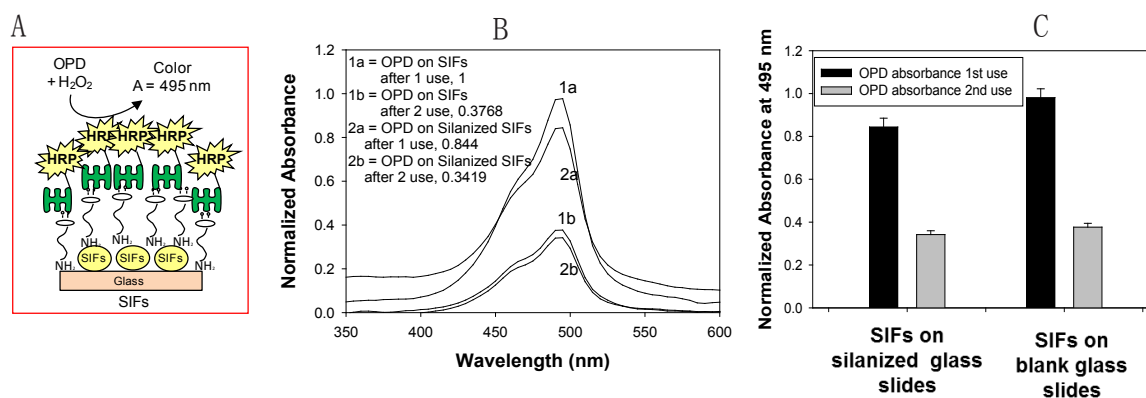
SIFs were deposited on blank glass slides and APTES-coated glass slides by using the method as described in our previous work [1]. These plasmonic surfaces were later characterized by measuring their absorbance spectrum. Fig. 1 shows the absorbance and normalized absorbance spectrum of the SIFs-deposited glass slide and SIFs-deposited APTES-coated glass slides. The maximum absorbance and wavelength recorded for SIFs-deposited glass slide and SIFs-deposited APTES-coated glass slides were (0.294, @430 nm) and (0.445, @400 nm) respectively (Fig. 1A). The normalized absorbance spectrum for SIFs on silanized glass slides



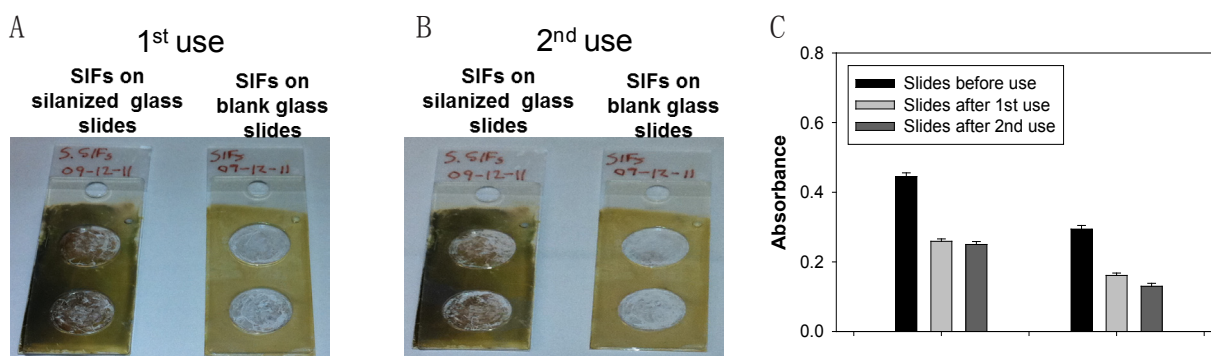
**Scheme. 1** Schematic depiction of Surface modification of SIFs with Biotin-PEG-Amine: HRP enzyme is deposited onto SIFs via Biotin-PEG-Amine / streptavidin interactions. Two different glass slides were used for the preparation of SIFs: blank glass slides and glass slides modified with 3-aminopropyltriethoxysilane, which contains terminal amine groups.



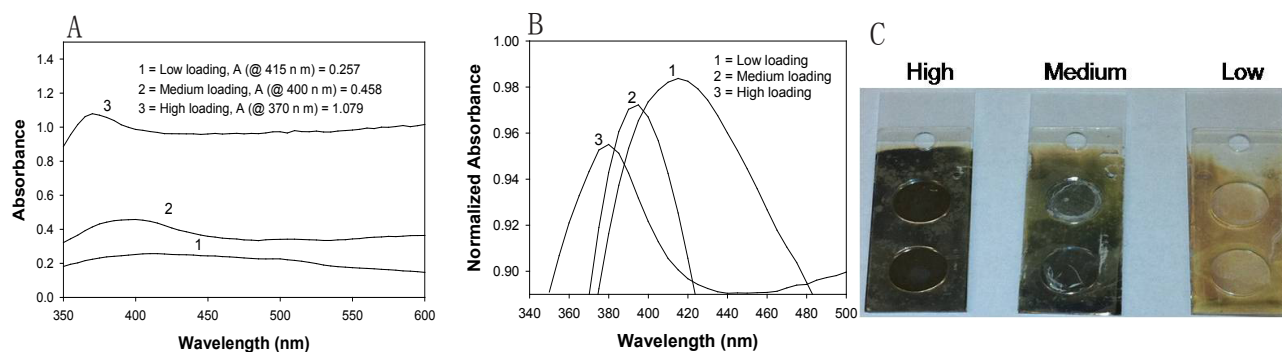
**Fig. 1** (A): Absorption spectrum for glass slides modified with 3-aminopropyltriethoxysilane (SIFs-deposited APTES-coated glass slide) and SIFs-deposited blank glass slides. (B): Normalized Absorbance for both surfaces. (C): Real-color photographs of both surfaces.



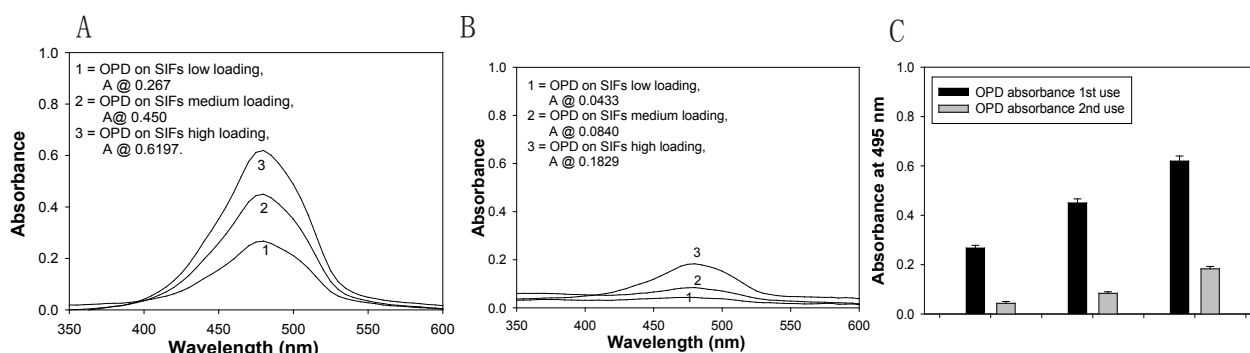
**Fig. 2** Schematic depiction of the new enzyme immobilization strategy employed in this study (A). Normalized absorbance spectrum of OPD at 495 nm (B) and (C) absorption of OPD on SIFs-deposited APTES-coated glass slide, and SIFs-deposited glass slide after 1st and 2nd use.



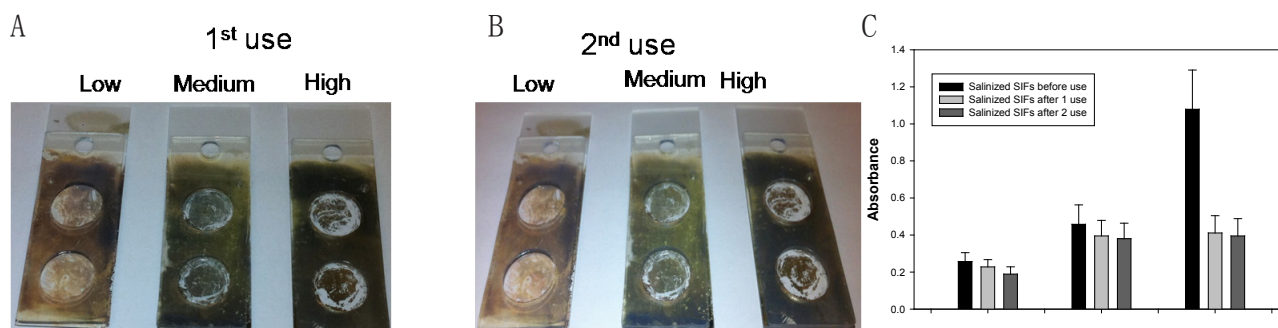
**Fig. 3** Real-color photographs of SIFs used in surface modification of SIFs with Biotin-PEG-Amine: SIFs-deposited APTES-coated glass slides and SIFs-deposited blank glass slide, (A) after 1st use, and (B) after 2nd use. (C) Corresponding absorbance values of SIFs.



**Fig. 4** (A) Absorption spectrum for SIFs-deposited APTES-coated glass slides: low, medium and high loading, (B) Normalized absorbance spectrum, (C) Real-color photographs.



**Fig. 5** Absorbance of OPD at 495 nm (A and B) and (C) Absorption spectrum of OPD on SIFs-deposited APTES-coated glass slide (low, medium and high loading) after 1st and 2nd use.



**Fig. 6** Real-color photographs of SIFs used in surface modification of SIFs with Biotin-PEG-Amine: low, medium and high loading (A) after 1st use, (B) after 2nd use. (C) Corresponding absorbance values of these SIFs at their relative surface plasmon resonance peaks.

shows a blue shift in the SPR peak and an increase in the surface plasmon resonance peak (SPR) despite the fact that identical preparation procedure was carried out for both surfaces (Fig. 1B). Fig. 1C shows the real color photograph of SIFs-deposited glass slides and SIFs-deposited APTES-coated glass slides.

It is important to discuss the reasons for the selection of the surface modification strategy in this work before the presentation of the data. The selected molecule, BEA, is a water-soluble compound that contains a terminal primary amine, biotin and polyethylene glycol chain, which gives the BEA a long and flexible connection to minimize steric hindrance involved with binding to avidin molecules [13-15]. The terminal amine group is used for the chemisorption of BEA to SIFs, while the biotin group

is used to attach HRP-streptavidin to SIFs surface. This assembly scheme places HRP~6 nm away from the silver surface (Fig. 2A). Since streptavidin is attached to the SIFs surface via the biotin groups on BEA, HRP is placed as the top layer of the assembly and involved in enzymatic conversion of OPD without steric hindrance from the molecules within the assembly.

The enzymatic activity was studied by placing a solution of OPD on the enzyme-modified surfaces of the SIFs-deposited blank glass slides and SIFs-deposited APTES-coated glass slides. The conversion of OPD by HRP into a colored product (from colorless to light brown) was followed by measuring absorption spectrum of the initial and final OPD solution. In this regard, the colored solution was removed after ~2 mins of incubation



and placed in a glass vial containing sulfuric acid to stop the conversion of OPD. The absorbance spectrum of the colored solution was then taken after two uses of the same surface. The absorption spectrum of OPD solution after 1<sup>st</sup> and 2<sup>nd</sup> use on both SIFs-deposited blank glass slides and SIFs-deposited APTES-coated glass slides is shown in Fig. 2B. The comparison of the enzymatic activity by both surfaces was carried out by plotting absorbance value at 495 nm after 1<sup>st</sup> and 2<sup>nd</sup> use (Fig. 2C), which shows a similar extent of enzymatic activity after 1<sup>st</sup> and 2<sup>nd</sup> use. During these experiments, we visually observed a significant loss of silver nanoparticles from both surfaces and carried out further experiments to determine the extent of loss of nanoparticles.

Fig. 3 shows the real-color photographs of SIFs-deposited blank glass slides and SIFs-deposited APTES-coated glass slides after the 1<sup>st</sup> and 2<sup>nd</sup> use, and Fig. 3C shows the corresponding absorbance values at their surface resonance peaks for both surfaces before use, after 1<sup>st</sup> and 2<sup>nd</sup> use. The decrease in absorbance from SIFs on blank glass slides after two uses (>50%) implies that these surfaces are unlikely to be re-usable for further repeated use. In contrast, there was a relatively smaller loss of silver nanoparticles from the SIFs-deposited APTES-coated glass slides (40%). Subsequently, further investigation for optimum enzymatic activity was carried out using SIFs-deposited APTES-coated glass slides, where the extent of silver nanoparticles was varied: low, medium and high loading.

Fig. 4A, Fig. 4B shows absorbance and normalized absorbance for SIFs with low, medium and high loading on APTES-coated glass slides. As observed and described previously [1], the surface resonance peak for SIFs show a blue shift as the loading of silver nanoparticles is increased. The real-color photographs of SIFs with low, medium and high loading on APTES-coated glass slides visually corroborate the increase in nanoparticle loading on SIFs.

Fig. 5 shows the absorbance spectrum of the colored solution obtained after the interaction of OPD and HRP-streptavidin for the different loading after 1<sup>st</sup> and 2<sup>nd</sup> use. The absorbance value at 495 nm for OPD measured for the SIFs with different loading after 1<sup>st</sup> use was increased as the loading of silver nanoparticles was increased: low loading ( $A_{495\text{ nm}}=0.265$ ), medium loading (0.450), and high loading (0.6197). The increase in absorbance of the different loading indicates that Biotin-PEG-Amine, which is used for the optimization of enzymatic activity on SIFs appears to improve the enzymatic activity as the loading is increased. When compared to our previous work where we used three different strategies for enzyme immobilization (Biotin-avidin, self-assembled monolayer hexamethylenediamine, and Poly-L-lysine monolayer [1]) to study the nanoparticle-enzyme distance- and nanoparticle loading-dependent enzymatic activity, the use of Biotin-PEG-Amine further improved the enzymatic activity. After the 2<sup>nd</sup> use of the SIFs with different

loading (Fig. 5B), we observed a decrease in the OPD absorbance spectrum, which indicates either the loss of enzyme activity and/or loss of SIFs from the glass surface due to multiple washing steps taking place after each step of the assay. Fig. 5C shows the absorbance of the colored solution at 495 nm after 1<sup>st</sup> and 2<sup>nd</sup> use for all loading, where 80%, 80% and 70% decrease in absorbance value for SIFs with low, medium and high loading, respectively. To further investigate whether the decrease in OPD absorbance was due to the potential detachment of SIFs from the surfaces as a result of extensive washing and/or loss of enzymatic activity, the absorbance spectrum and real-color photographs of SIFs after 1<sup>st</sup> and 2<sup>nd</sup> use were taken and shown in Fig. 6. Fig. 6 clearly shows that there was only a slight detachment of SIFs with low and medium loading from the surface after the 2 runs (<10%), which implies that the decrease in OPD absorbance on these surfaces was mainly due to the loss of enzymatic activity (and/or enzyme detachment). On the other hand, SIFs with high loading showed >60% decrease in absorbance for the surface plasmon resonance peak of SIFs, which implies that the decrease in OPD absorbance on these surfaces was mainly due to the detachment of SIFs from surfaces. Although not determined, it is thought that the loss of enzymatic activity also contributed to the decrease in the OPD absorbance. Based on the comparison of the data presented here with the data generated for other enzyme immobilization strategies in our previous work [1], we can conclude that the immobilization of enzyme onto SIFs using BEA as the linker yields the highest enzyme activity to date. Further investigation of other enzymes for plasmon-enhanced enzymatic reactions is ongoing in our laboratory and will be reported in due course.

## Conclusions

In this work, surface modification of SIFs was carried out using Biotin-PEG-Amine for the optimization of the plasmon-enhanced enzymatic activity. The enzyme (HRP) activity on several SIFs surfaces was assessed by absorption measurements. It was demonstrated that the enzymatic activity can be enhanced using SIFs-deposited blank glass slides and SIFs-deposited APTES-coated glass slides. However, significant losses of silver nanoparticles from SIFs-deposited blank glass slides were observed. Subsequently, we employed SIFs-deposited APTES-coated glass slides with different loading (low, medium and high) for further investigation. The enzymatic activity was also shown to be increased as the loading of SIFs on APTES-coated glass slides was increased. Biotin-PEG-Amine, which has a spacer arm (PEG) that places the enzyme 6 nm away from the silver surface, was shown to be the best option for the immobilization of enzymes on SIFs to date, which are employed in plasmon-enhanced enzymatic reactions.

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