Electronic Supplementary Material (ESI) for Analytical Methods.

Supporting Information for

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

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1. Characterization of gold thin films by Atomic Force Microscopy (AFM). The data shown in Figure 1 suggest the formation of aggregates of the plasmonic nanostructures on the glass slides to form a 5-10 nm thick semi-continuous and/or continuous film. To support these results, we further characterized the gold thin films (as an example thin film) using an AFM. For the sake of brevity, these results are provided in the Supporting Information (Figure S1-S3). As expected, the height of the gold thin films (1.26 ± 0.31 nm, 4.01 ± 1.27 nm, and 9.02 ± 1.34 nm) was found to correspond to the thickness of the films monitored by our sputter coater. The AFM images revealed that 1 nm thick gold thin films were deposited as semi-continuous film (with island-like structures) and 5 nm and 10 nm gold thin films were appears to be deposited as a continuous film. The nanoscale morphology of the gold films indicates the formation of a monolayer. Silver, copper and nickel films are expected to have similar features (data not shown) as described for gold thin films.

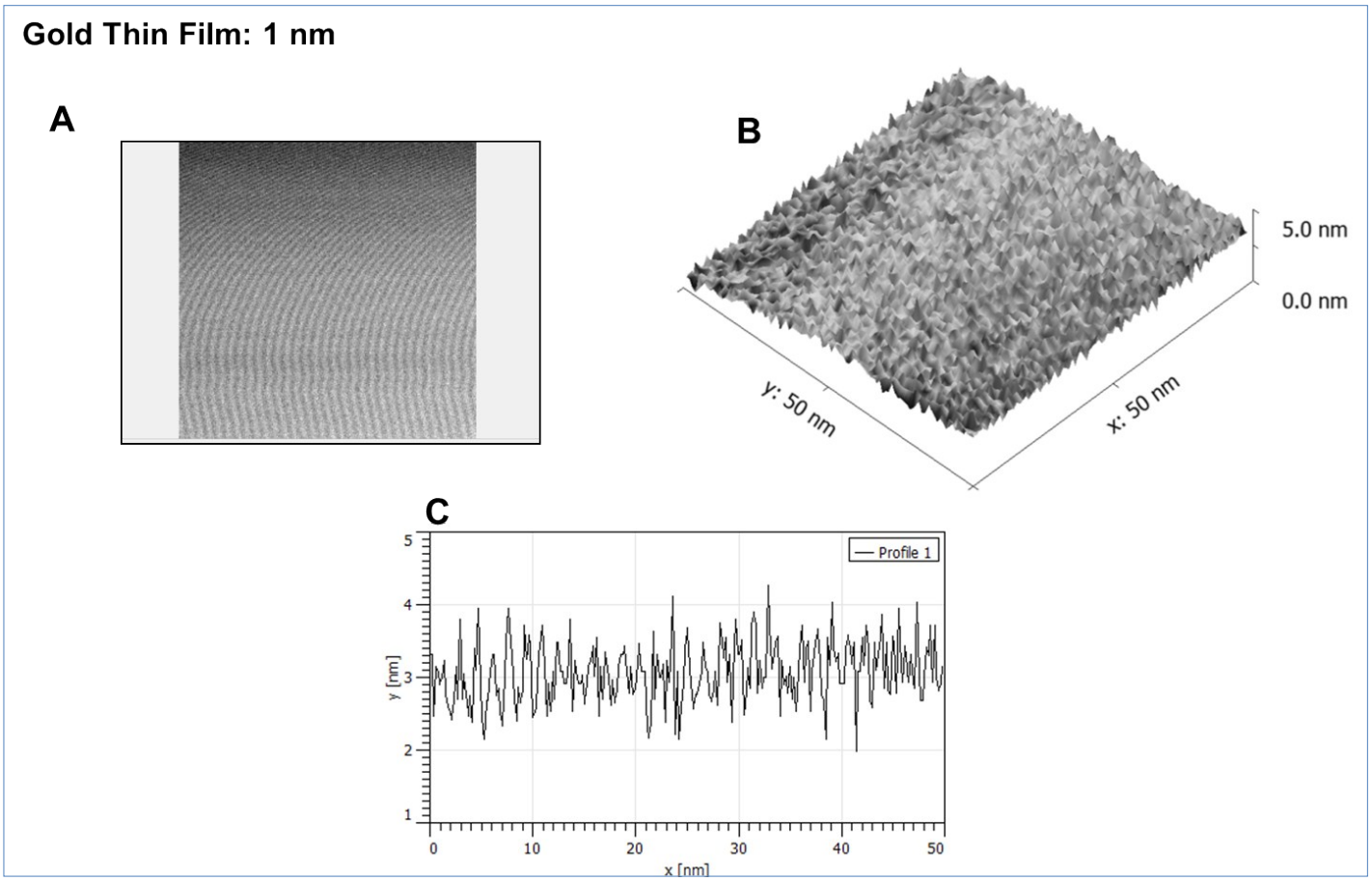


Figure S1. AFM analysis (phase image (**A**), 3D image (**B**), and line scan (**C**) displays of 1 nm thick gold thin films.

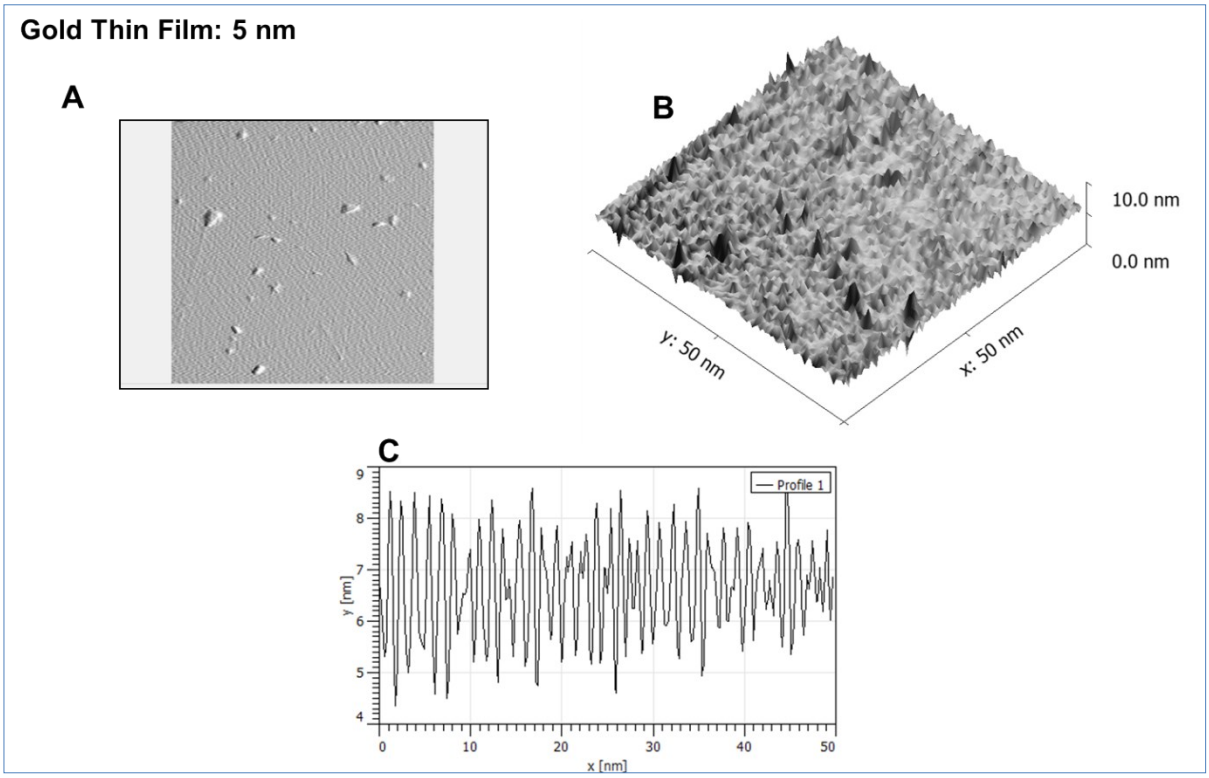


Figure S2. AFM analysis (phase image (**A**), 3D image (**B**), and line scan (**C**) displays of 5 nm thick gold thin films.

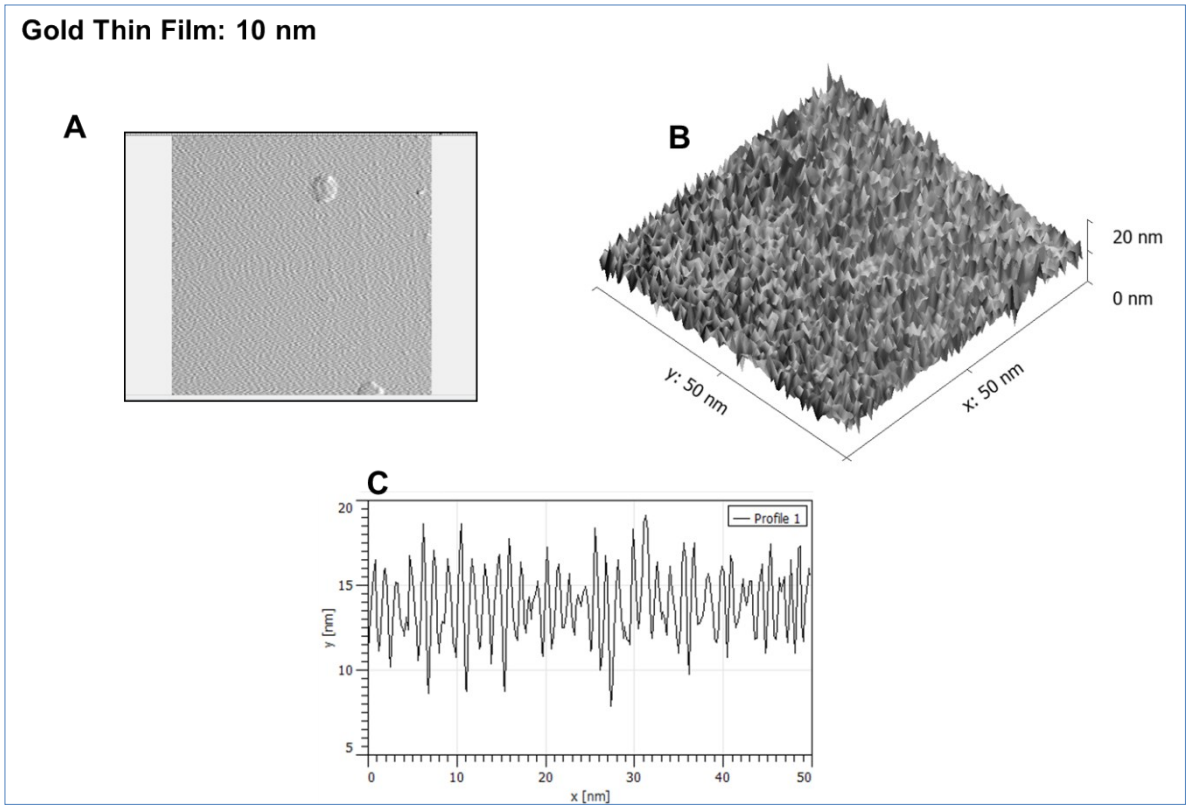


Figure S3. AFM analysis (phase image (A), 3D image (B), and line scan (C) displays of gold 10 nm thick gold thin films.

2. Colorimetric response of HRP and AP in model bioassays.

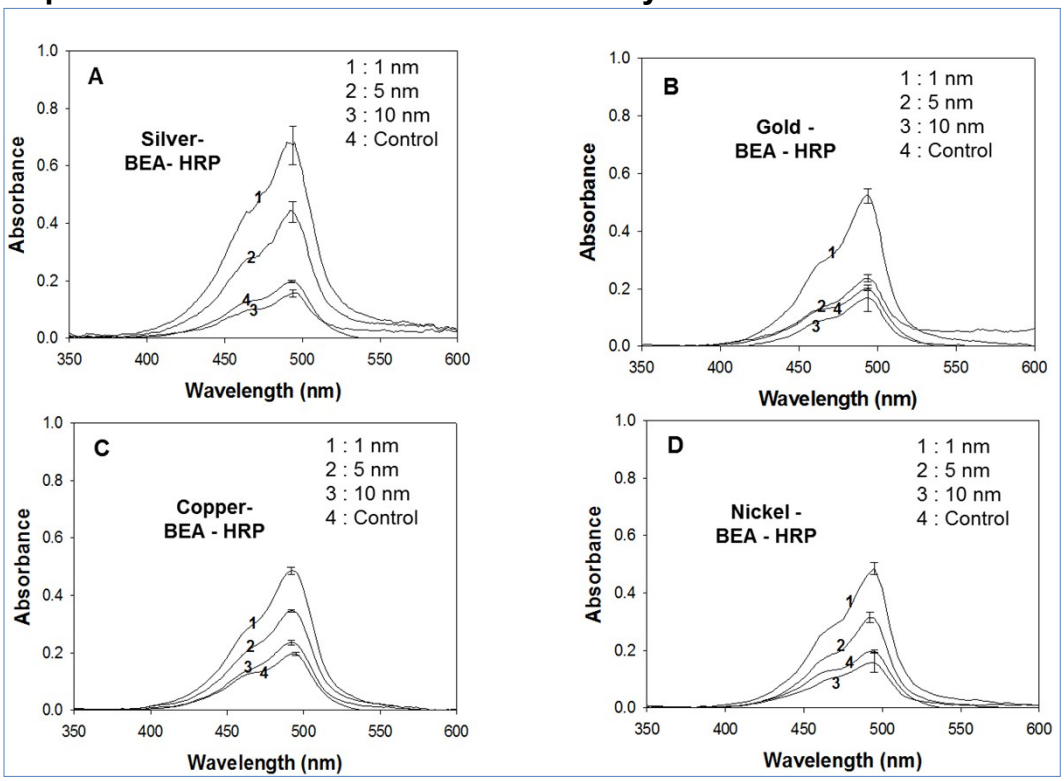
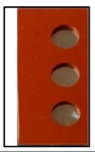


Figure S4. Colorimetric response of HRP on (**A**) silver, (**B**) gold, (**C**) copper, and (**D**) nickel thin films and on control surface (i.e. silanized glass without plasmonic thin films).

Table S1. Summary of results for enzymatic response for HRP and AP on plasmonic thin films (1 nm thick) in model bioassays. Abs: Absorbance.

Plasmonic Thin Films		Silver (1 nm) on glass	Gold (1 nm) on glass	Copper (1 nm) on glass	Nickel (1 nm) on glass	Silanized glass (Control: No plasmonic films)
I. Adsorbed Enzyme (ng/mm^2)	AP	18 ± 0.97	18 ± 1.5	19 ± 1.5	17 ± 1.0	18 ± 1.0
	HRP	23 ± 1.8	23 ± 2.4	21 ± 1.2	19 ± 6.4	18 ± 2.0
II. Total Colorimetric Response (Abs)	AP	0.30 ± 0.03	0.27 ± 0.01	0.23 ± 0.01	0.24 ± 0.02	0.24 ± 0.007
	HRP	0.67 ± 0.06	0.52 ± 0.02	0.48 ± 0.01	0.45 ± 0.05	0.21 ± 0.006
III. Non-Specific Enzymatic Response (Abs)	AP	0.11 ± 0.01	0.13 ± 0.02	0.10 ± 0.01	0.11 ± 0.01	0.07 ± 0.004
	HRP	0.22 ± 0.08	0.24 ± 0.01	0.28 ± 0.01	0.23 ± 0.01	0.10 ± 0.003
IV. Background (Abs)	AP	0.003 ± 0.001	0.003 ± 0.002	0.01 ± 0.01	0.002 ± 0.002	0.004 ± 0.001
	HRP	0.01 ± 0.003	0.03 ± 0.003	0.003 ± 0.007	0.001 ± 0.003	0.01 ± 0.001
V. Net Enzymatic Response (Abs: II – III – IV)	AP	0.187	0.137	0.120	0.128	0.166
	HRP	0.440	0.250	0.197	0.219	0.10
VI. Enhancement Factor (V for PNFs / V for control)	AP	**1.13**	**0.83**	**0.72**	**0.80**	-
	HRP	**4.4**	**2.50**	**1.97**	**2.19**	-

Table S2: Real-color photographs of gold, nickel, and copper-deposited glass slides of 1 nm, 5 nm, and 10 nm thick before and after model bioassays with HRP.

	Real-color photographs of plasmonic thin films before and after HRP reaction with OPD					
Thickness (nm)	1 nm		5 nm		10 nm	
	Before	After	Before	After	Before	After
Silver						
Gold						
Copper						
Nickel						

2. Colorimetric response of AP on plasmonic thin films. To investigate the effect of the extent of immobilized AP on the surface and colorimetric response, the extent of AP coverage on all plasmonic thin films and control sample were calculated. The protein coverage for all samples were comparable, these results can also be seen in Table S1: silver (18 ± 0.97 ng/mm^2), gold (18 ± 1.5 ng/mm^2), nickel (17 ± 1.0 ng/mm^2), copper (19 ± 1.5 ng/mm^2), and control sample (18 ± 1.0 ng/mm^2), respectively. The observation of low colorimetric response from 5 nm and 10 nm thick plasmonic thin films was thought to be due to the detachment of the enzyme and plasmonic thin films during incubation and wash steps. To confirm whether the result of low colorimetric response obtained is due to loss of enzymes/plasmonic thin films from the surface, digital images of the plasmonic thin films were taken before and after the AP reaction (Supporting Information, Table S3). Plasmonic thin films of 5 nm thickness were visually observed to lose a small amount of metal from the surface of silanized glass substrate after enzymatic reaction is carried out. On the other hand, 10 nm thick plasmonic thin films show a rapid loss of color, and this correlates with the observation of low colorimetric response. In addition to the results revealed from the digital images obtained, the extent of protein coverage for 5 nm and 10 nm thick for all plasmonic thin films were also determined (Supporting Information, Table S4-S5). The extent of AP coverage on 5 nm and 10 nm thick plasmonic thin films can be attributed to the observation of low colorimetric response from these surfaces.

To quantitate the effect of plasmonic thin films on the colorimetric response of AP as compared to the control sample, the enhancement factors of the colorimetric response obtained for AP was calculated and is shown in Table S1, S4, and S5, (Supporting Information). Table S1 revealed that colorimetric response of AP was enhanced by 1.13-fold for silver thin films, and was decreased to 0.83-fold for gold, 0.72-fold for copper, and 0.80-fold for nickel thin films (all 1 nm thick) as compared with the control sample. [Note: control experiment, where BEA-3400 Da was omitted from the surface before the attachment of HRP or AP revealed that the background signal on plasmonic thin films were slightly larger than on control samples (Supporting Information, Figure S6)]. This implies that BEA-3400 Da, which places the enzyme ~6 nm form the plasmonic surface, plays a vital role in the enhancement of enzymatic colorimetric response.

Table S3: Real-color photographs of gold, nickel, and copper-deposited glass of 1 nm, 5 nm, and 10 nm thick before and after model bioassays with AP.

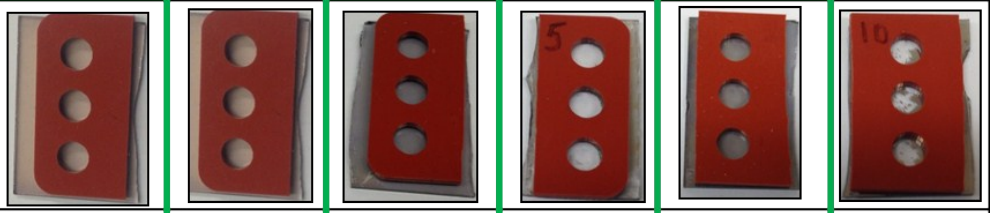
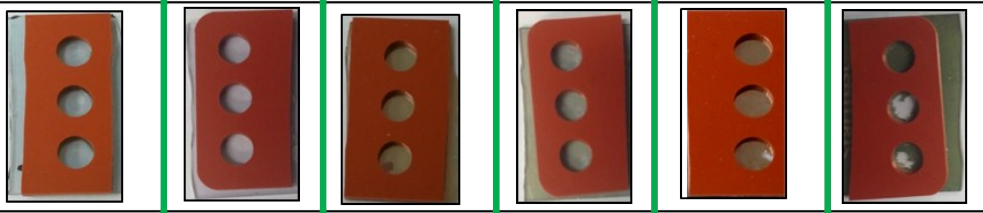
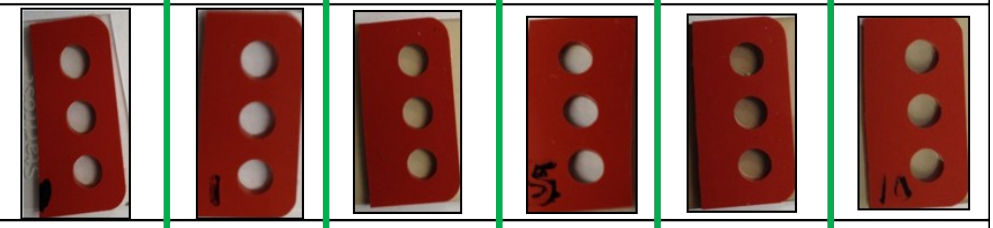
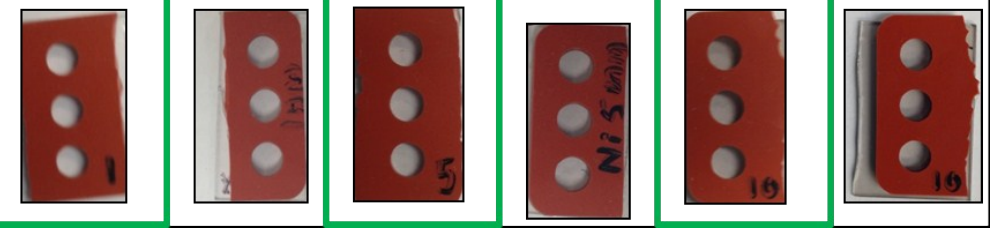
	Real-color photographs of plasmonic thin films before and after AP reaction with pNPP					
Thickness (nm)	**1 nm**		**5 nm**		**10 nm**	
	Before	After	Before	After	Before	After
Silver						
Gold						
Copper						
Nickel						

Table S4. Summary of results for enzymatic response for HRP and AP on plasmonic thin films (5 nm thick) in model bioassays. Abs: Absorbance

Plasmonic Thin Films		Silver (5 nm) on glass	Gold (5 nm) on glass	Copper (5 nm) on glass	Nickel (5 nm) on glass	Silanized glass (Control: No plasmonic films)
I. Adsorbed Enzyme (ng/mm^2)	AP	9 ± 0.21	4 ± 1.56	5 ± 2.63	8 ± 0.28	18 ± 1.0
	HRP	16 ± 1.14	9 ± 3.00	12 ± 2.42	11 ± 1.30	18 ± 2.0
II. Colorimetric Response (Abs)	AP	0.25 ± 0.03	0.14 ± 0.005	0.18 ± 0.03	0.21 ± 0.02	0.24 ± 0.007
	HRP	0.44 ± 0.04	0.23 ± 0.01	0.35 ± 0.009	0.31 ± 0.02	0.21 ± 0.006
III. Non-Specific Enzyme Reaction (Abs)	AP	0.061 ± 0.004	0.063 ± 0.002	0.10 ± 0.003	0.06 ± 0.004	0.07 ± 0.004
	HRP	0.13 ± 0.008	0.09 ± 0.001	0.11 ± 0.008	0.13 ± 0.004	0.10 ± 0.003
IV. Background (Abs)	AP	0.029 ± 0.004	0.015 ± 0.003	0.035 ± 0.005	0.029 ± 0.001	0.004 ± 0.001
	HRP	0.0063 ± 0.0006	0.0033 ± 0.0003	0.0064 ± 0.0008	0.0054 ± 0.0001	0.01 ± 0.001
V. Net Enzyme Reaction (Abs: II-III-IV)	AP	0.16	0.06	0.045	0.121	0.166
	HRP	0.30	0.14	0.233	0.174	0.10
VI. Enhancement Factor (V for PNFs / V for control)	AP	0.96	0.37	0.27	0.72	-
	HRP	3.0	1.4	2.33	1.74	-

Table S5. Summary of results for enzymatic response for HRP and AP on plasmonic thin films (10 nm thick) model bioassays. Abs: Absorbance.

Plasmonic Thin Films		Silver (10 nm) on glass	Gold (10 nm) on glass	Copper (10 nm) on glass	Nickel (10 nm) on glass	Silanized glass (Control: No plasmonic films)
I. Adsorbed Enzyme (ng/mm^2)	AP	5 ± 0.89	1 ± 0.54	3 ± 0.53	2 ± 0.79	18 ± 1.0
	HRP	3 ± 0.57	3 ± 0.79	4 ± 1.03	3 ± 0.37	18 ± 2.0
II. Colorimetric Response (Abs)	AP	0.17 ± 0.02	0.10 ± 0.005	0.11 ± 0.006	0.10 ± 0.007	0.24 ± 0.007
	HRP	0.16 ± 0.01	0.16 ± 0.05	0.24 ± 0.009	0.16 ± 0.005	0.21 ± 0.006
III. Non-Specific Enzyme Reaction (Abs)	AP	0.04 ± 0.003	0.07 ± 0.003	0.05 ± 0.005	0.03 ± 0.009	0.07 ± 0.004
	HRP	0.08 ± 0.005	0.05 ± 0.003	0.09 ± 0.009	0.08 ± 0.004	0.10 ± 0.003
IV. Background (Abs)	AP	0.004 ± 0.0007	0.008 ± 0.0003	0.014 ± 0.003	0.005 ± 0.001	0.004 ± 0.001
	HRP	0.003 ± 0.0008	0.006 ± 0.0004	0.006 ± 0.0005	0.004 ± 0.0005	0.01 ± 0.001
V. Net Enzyme Reaction (Abs: II-III-IV)	AP	0.126	0.022	0.046	0.065	0.166
	HRP	0.077	0.104	0.144	0.076	0.10
VI. Enhancement Factor (V for PNFs / V for control)	AP	0.76	0.13	0.27	0.39	-
	HRP	0.77	1.04	1.44	0.76	-

Figure S5. Colorimetric response of AP on (A) silver, (B) gold, (C) copper, and (D) nickel thin films and on control surface (i.e. silanized glass without plasmonic thin films) model bioassays.

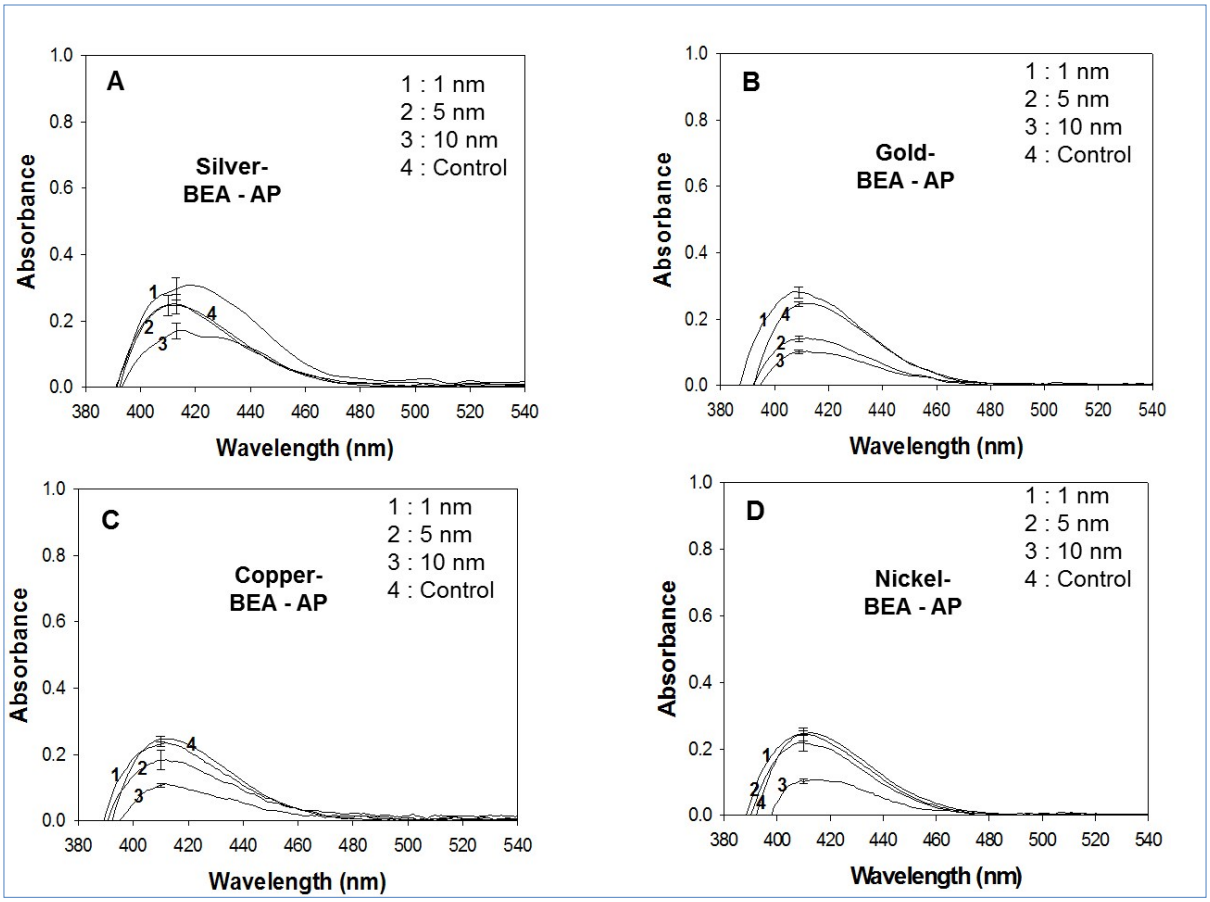
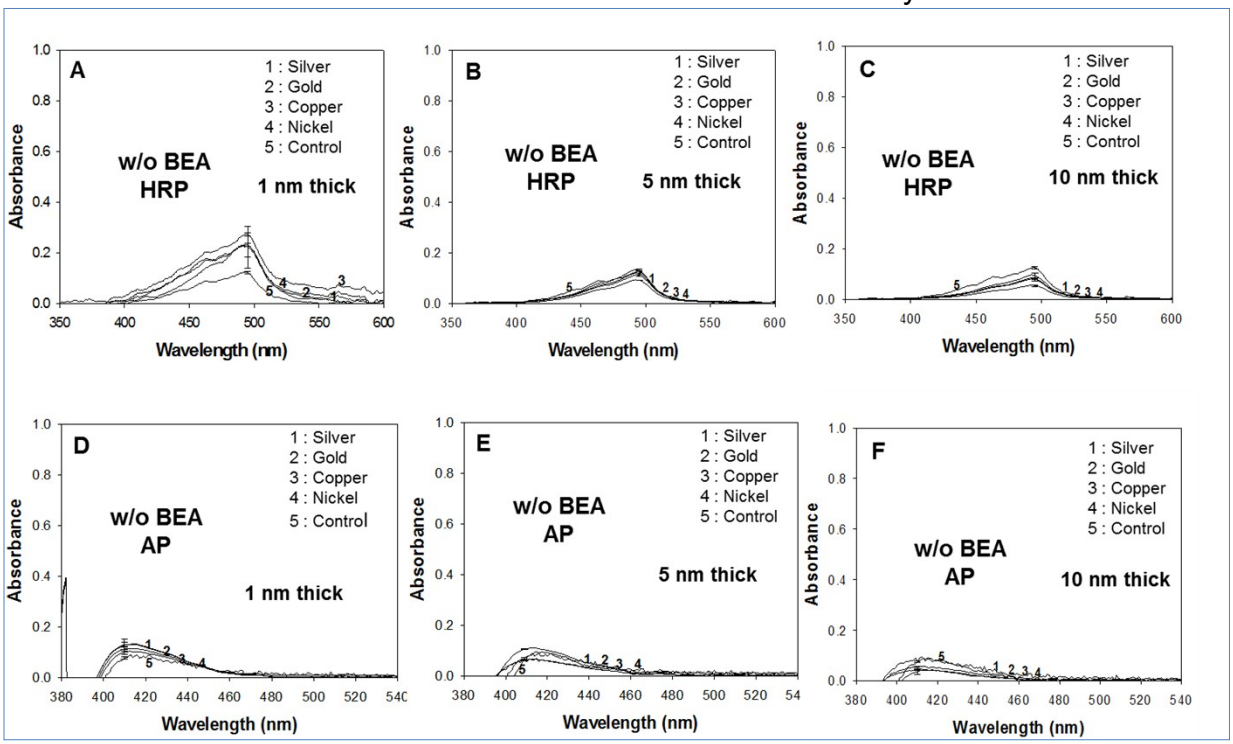


Figure S6. Adsorption spectrum of HRP (A-C) and AP (D-F) reaction on PNFs of 1 nm, 5 nm, and 10 nm thick w/o BEA-3400 Da model bioassays.



3. The effect of the extent of immobilized HRP on the colorimetric response in bioassays for GFAP. In this regard, the extent of HRP on 1 nm thick silver thin films were determined by measuring absorbance at 280 nm are shown in Table S6. It is important to note that the measured absorbance value includes absorbance for all proteins on the surface (protein A, capture antibody, GFAP and secondary antibody and HRP-labeled polyclonal antibody as shown in Scheme 2). Since all surfaces were incubated with the same initial solutions of protein A and capture antibody, the contribution of these proteins to the overall absorbance value at 280 nm on all surfaces are expected to be identical.

On the other hand, the contribution of HRP to the overall absorbance value at 280 nm on all surfaces varies with the amount of GFAP present on the surface. Subsequently, the extent of HRP presented in Table S6 is calculated based on the difference in the absorbance value at 280 nm measured from the surface and the HRP-labeled polyclonal antibody solution removed from the surfaces after its incubation. Since the surface area and the volume of each well are known, we converted the absorbance value to the amount of adsorbed HRP per surface area of each well.

The observation of the detachment of silver thin films from surface was also investigated using digital photography (Table S7, Supporting Information), which show slight loss of color, corroborating the spectroscopic observations described above. To assess the loss of enzymes from the silver thin films after the last step (conversion of OPD to the colored product) of the bioassays are completed, we subsequently measured the absorbance of the colored product in the 240-600 nm region of the electromagnetic spectrum and results are shown in Figure S8 (Supporting Information). Figure S8 shows the absorbance spectrum (240–600 nm) of colored product after enzymatic reaction of HRP immobilized on bioassays revealed a peak absorption value at 495 nm for the colored product on silver thin films which increases as the concentration of GFAP is increased as described in the main text.

Table S6. Summary of results for adsorbed HRP in standard bioassays and modified standard bioassays for GFAP at different concentrations (1-100 ng/mL).

Concentration of GFAP in solution (ng/mL)	Standard Bioassay: Adsorbed Enzyme (HRP) on Silver-GFAP (ng/mm^2)	Modified Standard Bioassay: Adsorbed Enzyme (HRP) on Silver-GFAP-BEA (ng/mm^2)
1	14 ± 8.2	20 ± 4.0
5	17 ± 8.5	22 ± 7.0
10	19 ± 9.2	24 ± 8.0
25	24 ± 10.3	28 ± 4.0
50	28 ± 9.8	32 ± 4.0
100	31 ± 4.4	36 ± 6.0

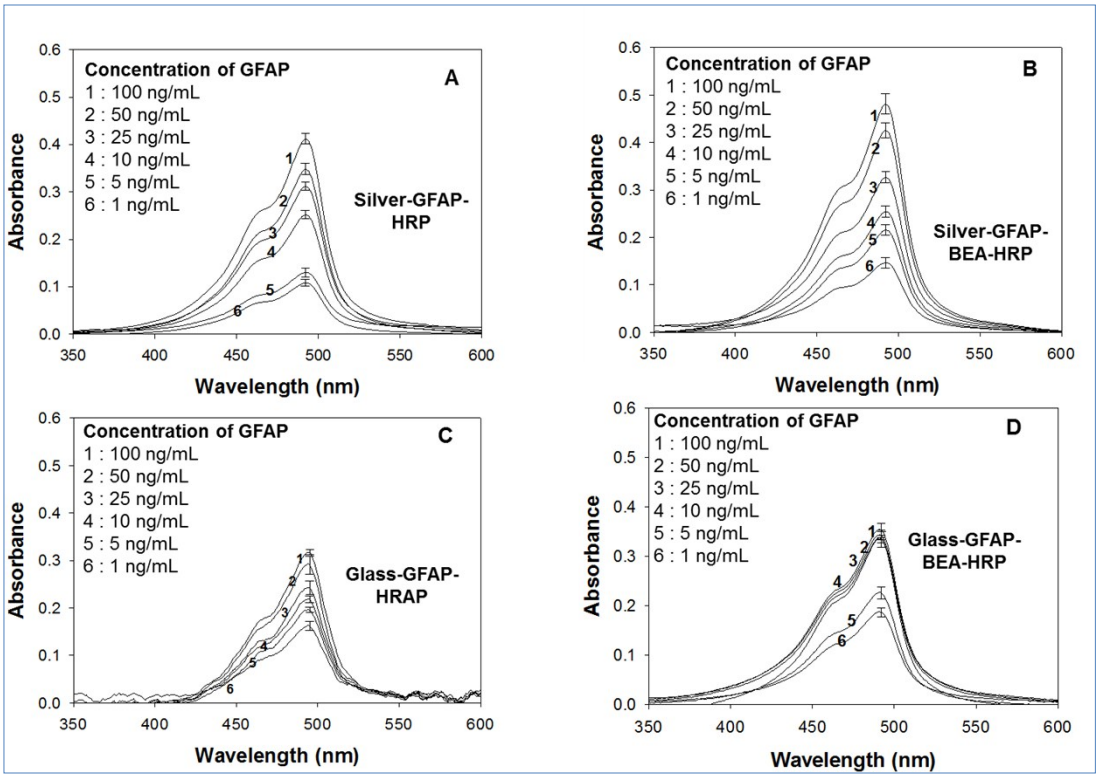
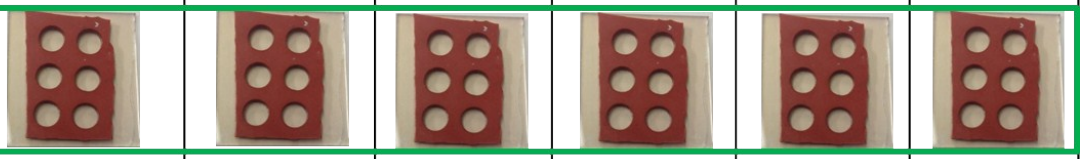
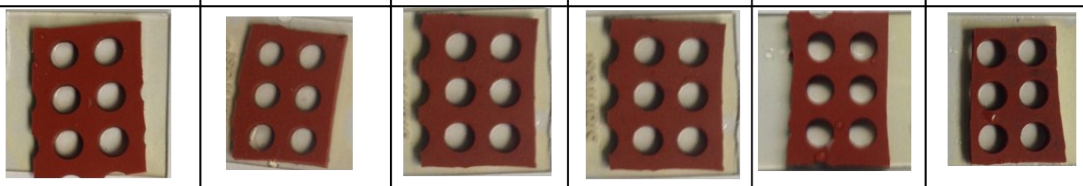
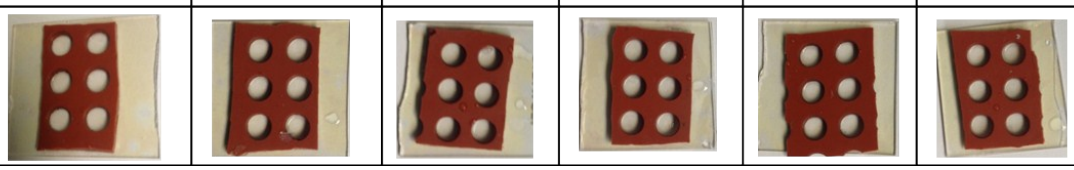


Figure S7. Colorimetric response for HRP immobilized in bioassays for GFAP at different concentrations (1-100 ng/mL) on (A) standard bioassays on silver thin films, (B) modified standard bioassays on silver thin films, (C) standard bioassays on glass, and (D) modified standard bioassays on glass.

Table S7: Real-color photographs of silver before and after standard bioassays and modified standard bioassays for GFAP at different concentrations (1-100 ng/mL).

Concentration of GFAP (ng/mL) →		1	5	10	25	50	100
SNFs	Before						
Silver-GFAP-HRP	After						
Silver-GFAP-BEA-HRP	After						

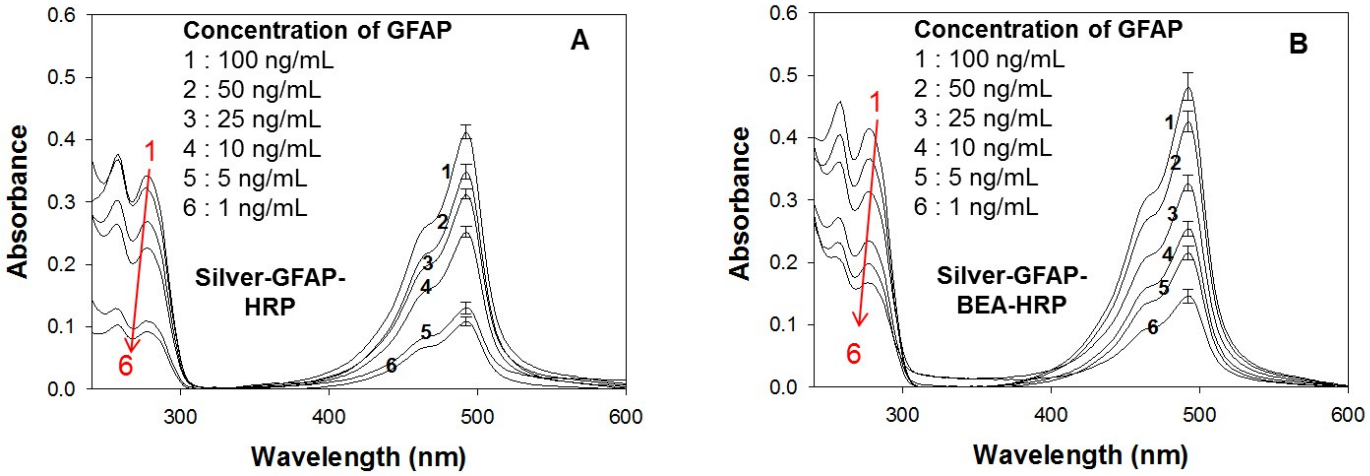


Figure S8. Shows the absorbance spectrum (240 – 600 nm) of colored product in bioassays for GFAP concentrations (1-100 ng/mL): (A) standard bioassays on silver on silver thin films and (B) modified standard bioassays on silver thin films.