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1 **Gold Nanobipyramids integrated Ultrasensitive Optical and Electrochemical Biosensor**  
2 **for Aflatoxin B<sub>1</sub> Detection**

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9  
10 **Abstract**

11 This work reports the development of an electrical and optical biosensing for label-free  
12 detection of Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) using gold (Au) nanobipyramids (NBPs). AuNBPs were  
13 synthesized through a two-step seed-mediated growth process followed by an exchange of  
14 capping agent from surfactant to lipoic acid. Pure and monodispersed AuNBPs of 70 nm base  
15 length were obtained and deposited on indium tin oxide (ITO)-coated glass substrate  
16 modified with self-assembled (3-Aminopropyl) triethoxysilane (APTES) film. The  
17 characterization of the obtained surfaces using spectroscopy, microscopy and diffractometry  
18 confirms the formation of AuNBPs, the conjugation to ITO electrode substrate and the  
19 immobilization of anti-AFB<sub>1</sub> antibodies. AuNBPs modified ITO substrates were used for  
20 both electrochemical and Surface Plasmon Resonance biosensing studies. Localized Surface  
21 Plasmon Resonance (LSPR) local field enhancement was demonstrated. SPR based AFB<sub>1</sub>  
22 detection was found to be linear in the 0.1 to 500 nM range with a limit of detection of 0.4  
23 nM, whereas, impedimetric AFB<sub>1</sub> detection was shown to be linear in the 0.1 to 25 nM range  
24 with a limit of detection of 0.1 nM. The practical utility of the impedimetric sensor was tested  
25 in spiked maize samples and 95-100% recovery percentage were found together with low  
26 relative standard deviation, proof of the robustness of this AFB<sub>1</sub> sensor.

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28  
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30  
31 **Keywords:** Gold nanobipyramids, Surface Plasmon Resonance, Electrochemical Impedance  
32 Spectroscopy, food toxin, Aflatoxin B<sub>1</sub>, Biosensor

33  
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## 35 **1. Introduction**

36 From the last few years, metallic nanoparticles (NPs) integrated plasmonic biosensors have  
37 gained considerable attention for label-free bioanalytical applications due to their high  
38 sensibility to changes in the wavelength form or surface angle [1, 2]. Real-time kinetic study  
39 of unlabelled biomolecules and binding/unbinding events are easily monitored by SPR  
40 technique [3]. Au is here one of the most well-known material for NP-based applications  
41 since the obtained NPs exhibit unique structural, electronic, magnetic and optical properties  
42 [4]. Moreover, in the field of sensing applications, the anisotropy of Au nanostructures  
43 integrated within SPR systems was shown to lead to enhanced biosensor performances [5].

44 Many efforts have been also made to decorate Au nanostructures over chip surface, in an  
45 attempt to generate SPR biosensor with amplified sensing output signal. Intriguingly,  
46 spherical shaped AuNPs have been extensively used whereas non-spherical Au  
47 nanostructures were neglected [6]. However, spherical AuNPs display only one SPR band,  
48 usually around 520 nm, while nanobipyramids (NBPs) for example, exhibit two SPR bands  
49 around 550 nm and 700 nm, due to elongated transverse and longitudinal directions sharing  
50 the same base.

51 Au systems with tailored shape, i.e. AuNBPs, are known to be more sensitive to refractive  
52 index change but also to trigger multi-fold electrochemical signal enhancement [6]. Thus, Wu  
53 et al. developed sensitive SPR biosensor based on graphene oxide modified AuNBPs for  
54 rabbit IgG detection [7]. The obtained limit of quantification was then 16-fold lower than  
55 when using conventional Au film surface. Efficient signal amplification of AuNBPs was then  
56 shown to contribute to sensitivity enhancement of SPR biosensor. Another work was also  
57 reported by Zhang et al. using AuNBPs and Au nanorods used for SPR and square wave  
58 voltammetry biosensors for the detection of immunoglobins [8]. The authors demonstrated  
59 here that AuNBP-based sensors exhibited a 64-fold enhanced sensitivity whereas Au  
60 nanorods system display only a 16-fold enhanced sensitivity signal, when compared to Au  
61 film surface. This positive effect is believed to be due to the AuNBPs sharp apexes which  
62 produce a stronger electromagnetic field than Au nanorods over the chip surface [7, 9].  
63 Similarly, in the field of electrochemical biosensors, nanostructure-based materials were also  
64 used to increase the detection efficiency thanks to their large surface-to-volume ratio, their  
65 improved electrical conductivity and their fast heterogeneous electron transfer rate [10].

66 In the present study, AuNBPs were used to develop both SPRi-based and electrochemical-  
67 based biosensors for Aflatoxin B<sub>1</sub>. This application is driven by the fact that aflatoxin-

68 contaminated food and feed products are responsible for hepatotoxic, hepatocarcinogenic and  
69 severe chronic and acute diseases [11, 12]. Available methods for Aflatoxin B<sub>1</sub> detection and  
70 quantification are high-pressure liquid chromatography, thin-layer chromatography,  
71 fluoroimmunoassay test, etc. Although some of them have attained high sensibility, the  
72 existence of certain limitations restricts their utility for routine food sample analysis such as  
73 trained personnel, expensive laboratory equipment and time-consuming [13].

74 Biosensing performances specifically depend upon construction strategy as well as the  
75 process to conjugate the biomolecules with the Au nanostructures' surface. In recent years,  
76 many efforts have been devoted towards the covalent decoration of anisotropic shape of Au  
77 nanostructures on transparent ITO coated-glass substrate. In the present work, ITO-based  
78 electrode has been fabricated by *in-situ* surface modification using EDC-NHS crosslinker  
79 followed by immobilization of anti-AFB<sub>1</sub> and passivation. Correspondingly, ITO-electrode  
80 surface has been constructed for electrochemical studies. SPR and electrochemical  
81 impedance spectroscopy (EIS) techniques have been used to carry out the biosensing studies  
82 for AFB<sub>1</sub> detection.

83

## 84 **2. Materials and Methods**

### 85 **2.1 Chemicals**

86 Cetyltrimethylammonium bromide (CTAB), Dichloromethane (DCM), Sulphuric acid  
87 (H<sub>2</sub>SO<sub>4</sub>), Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>), (3-Aminopropyl)triethoxysilane (APTES), Toluene,  
88 Chloroplatinic acid (H<sub>2</sub>PtCl<sub>6</sub>), Sodium Borohydride (NaBH<sub>4</sub>), Ethanolamine, Hydrochloric  
89 acid (HCl), Gold(III) chloride (HAuCl<sub>4</sub>), N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide  
90 hydrochloride (EDC), N-Hydroxy succinimide (NHS), Anti-aflatoxin B<sub>1</sub> antibodies (anti-  
91 AFB<sub>1</sub>) produced in rabbit and AFB<sub>1</sub> from *Aspergillus flavus* were purchased from Sigma-  
92 Aldrich (France). Lipoic acid was obtained from RepliGen (USA). All other chemical  
93 reagents were of analytical grade and used as such without further purification. Milli-Q  
94 purified water resistivity of 18.2 MΩ cm was used for the preparation of the aqueous  
95 solution.

### 96 **2.2 Instruments**

97 The UV-visible spectroscopy technique was used to carry out the optical measurements of  
98 synthesized AuNPs and AuNBPs using UV-visible spectrophotometer (Jasco V-730).  
99 Diffraction patterns of AuNBPs were measured using Rigaku X-ray diffractometer consisting  
100 of Cu K $\alpha$  radiation. FT-IR studies were performed using Perkin-Elmer Spectrum BXII

101 spectrophotometer. Transmission Electron Microscopy (TEM) images of synthesized AuNCs  
102 and AuNBPs were collected from Transmission Electron Microscopy (TEM) (Philips  
103 CM120). Surface morphology was examined by Scanning Electron Microscopy (SEM)  
104 (MEB Quanta 250). Atomic Force Microscopy (AFM, Integra, NT-MDT) was used to  
105 characterize AuNBPs electrode surface under contact force mode with standard golden-  
106 silicon cantilevers. Stepwise surface modification was characterized by contact angle  
107 measurements using contact angle meter (Data Physics OCA15EC). SPRi measurements  
108 were carried out using the SPRi+ Lab instrument (Horiba, France). Electrochemical  
109 measurements were performed using electrochemical analyzer Autolab  
110 Potentiostat/Galvanostat (Eco Chemie, The Netherlands) in PBS buffer (50 mM, 0.9%NaCl)  
111 containing in addition 5 mM of  $[\text{Fe}(\text{CN})_6]^{3-/4-}$ .

112

### 113 **2.3 Synthesis of AuNBPs**

114 AuNBPs were synthesized through a seed-mediated method using a previously reported  
115 procedure and slight modifications [14]. Briefly, seed solution of AuNPs was prepared in a  
116 reaction mixture composed of  $\text{HAuCl}_4$  (0.01M, 150  $\mu\text{L}$ ) and  $\text{H}_2\text{PtCl}_6$  (0.01M, 100  $\mu\text{L}$ ) with  
117 ice-cold freshly prepared  $\text{NaBH}_4$  (0.01 M, 900  $\mu\text{L}$ ) in CTAB (0.1 M, 9.75 mL) aqueous  
118 solution. In the first hour, the solution colour changed from orange to brownish-yellow,  
119 indicating the formation of seed AuNPs. To complete the reaction and the formation of  
120 AuNPs, the solution was kept at room temperature under light tight condition for 24 hours.  
121 Finally, the CTAB excess as well as impurities in the reaction mixture were removed by  
122 centrifugation at 5000 rpm for 20 minutes and washing of the obtained pellet several times  
123 with distilled water.

124 For the preparation of AuNBPs, purified Au seed particles (300  $\mu\text{L}$ ) were added into the  
125 growth solution prepared by the successive addition of  $\text{HAuCl}_4$  (0.01M, 1.75 mL),  $\text{H}_2\text{PtCl}_6$   
126 (0.01M, 50  $\mu\text{L}$ ),  $\text{AgNO}_3$  (0.01M, 400  $\mu\text{L}$ ), ascorbic acid (0.1 M, 320  $\mu\text{L}$ ) and HCl (1M, 600  
127  $\mu\text{L}$ ) in CTAB (0.1M, 40 mL) aqueous solution. The solution was kept at room temperature  
128 under light tight condition for 24 hours to complete the growth of AuNBPs. Meanwhile, the  
129 time interval UV-visible spectrum studies were carried out to follow the growth of AuNBPs.  
130 The resulting solution was purified by centrifugation at 8000 rpm for 30 minutes to remove  
131 excess solvent and the obtained pellet washed 3-time with distilled water.

132 Stepwise exchange from surfactant CTAB to lipoic acid was performed using ligand-  
133 exchange method while maintaining colloidal stability of AuNBPs [15]. To carry out this  
134 replacement, an equal ratio of CTAB-AuNBPs and DCM organic solvent was poured in a

135 vial and left to equilibrate for 2 hours at room temperature under light tight condition. Then,  
136 the colloidal solution of AuNBPs was carefully removed from the above reaction mixture and  
137 simultaneously injected into Lipoic acid (0.1 M) solution prepared in ethanol. The solution  
138 was kept for 2 hours at the room temperature. As-prepared Lipoic acid-AuNBPs were  
139 purified by centrifugation at 8000 rpm for 20 minutes. Prepared AuNBPs solution stored at  
140 4°C.

141

#### 142 **2.4 Preparation of AuNBPs electrodes**

143 ITO-coated glass electrodes ( $2 \times 1 \text{ cm}^2$ ) were cleaned by distilled water and rinsed with  
144 ethanol. ITO slides were dipped into a reaction mixture of  $\text{H}_2\text{O}_2/\text{NH}_3/\text{H}_2\text{O}$  in 1:1:5 ratio v/v/v  
145 and heated at 80°C for 30 minutes to achieve surface oxidation. Then, slides were dried at  
146 room temperature [16]. To obtain APTES self-assembled monolayer on ITO-coated glass  
147 surface, cleaned electrodes were functionalized with alkoxy silane molecules of  
148 APTES/toluene (0.2% v/v) for two hours and then washed with toluene to remove unbound  
149 APTES molecules [17]. Further, APTES/ITO electrodes were dipped overnight in the  
150 AuNBPs solution containing EDC (0.2M): NHS (0.05M) mixture. The carboxylic groups of  
151 lipoic acid-AuNBPs were therefore conjugated to amine terminal group of APTES. The  
152 surface-modified AuNBPs/SAM/ITO electrodes were rinsed with distilled water to remove  
153 the unbound AuNBPs molecules and dried at room temperature.

154

#### 155 **2.5 *In-situ* surface modification of functionalized electrode for SPRi studies**

156 To carry out the SPRi studies for AFB<sub>1</sub> detection, self-assembled amine-functionalized  
157 AuNBPs decorated electrode was first loaded into the SPRi system. The *in-situ* surface  
158 activation of the remaining lipoic acid was then performed in the presence of EDC (0.2M):  
159 NHS (0.05M) mixture under static condition for 7 minutes. Remaining solution over the  
160 activated electrode surface was removed and cleaned with PBS. Sequentially, 100 µg/mL of  
161 anti-AFB<sub>1</sub> was poured over the above-activated electrode and kept for another 7 minutes for  
162 covalent attachment of antibodies to the activated AuNBPs. Electrode surface was washed  
163 with PBS to remove the unbound antibodies and further treated with ethanolamine (1M in  
164 HCl, pH 8.5) to passivate the remaining activated groups. Phosphate buffer (pH 7.4) was used  
165 as a running buffer for all SPRi studies. All SPRi measurements were carried out at 25°C.

#### 166 **2.6 Immobilization of anti-AFB<sub>1</sub> on AuNBPs/SAM/ITO surface for electrochemical** 167 **studies**

168 Prior to immobilization of anti-AFB<sub>1</sub>, the surface-modified AuNBPs/SAM/ITO electrodes  
169 were activated using 10 μL of a EDC (0.2M): NHS (0.05M) mixture, poured on the electrode  
170 and kept in a humid chamber for 1 hour at room temperature. Later on, 10 μL of anti-AFB<sub>1</sub> at  
171 a concentration of 100 μg/mL were incubated for 1 hour on the activated surface under humid  
172 conditions at room temperature. Finally, the electrode activated remaining groups were  
173 passivated in the presence of ethanolamine (pH 8.5) and stored at 4°C.

## 174 **2.7 Real samples preparation**

175 Non-contaminated maize samples were purchased from the local market, and solutions were  
176 prepared using the previously reported method [18]. Briefly, 1 g of dried maize was spiked  
177 with 200 μL AFB<sub>1</sub> solution (different concentrations were prepared in PBS buffer) and left at  
178 room temperature for 1 hour. 20 mL of 80/20 (v/v) methanol/water solution was then added  
179 to the spiked maize and gently stirred on magnetic stirring for 2 hours under room  
180 temperature. To remove suspended particles and excess of solvent, centrifugation was  
181 performed at 5000 rpm for 30 minutes. The supernatant was carefully collected, and further  
182 dilutions were made with PBS (1:5). 200 μL of these solutions were used for the  
183 electrochemical measurements.

## 184 **3. Results and Discussion**

185 AuNBPs were prepared using a two-step seed-mediated growth process where H<sub>2</sub>PtCl<sub>6</sub> was  
186 used as shape directing agents, NaBH<sub>4</sub> as reducing agent and CTAB as an easy to use ionic  
187 stabilizing surfactant. **Figure 1(a) (step 1-3)** illustrates the stepwise synthesis of AuNBPs  
188 from AuNPs together with the ligand exchange process. **Figure 1(b) (step 4)** depicts the  
189 immobilization of AuNBPs over the APTES-modified ITO surface followed by activation  
190 and immobilization of anti-AFB<sub>1</sub> antibodies.

### 191 **3.1 Structural and Morphological studies**

192 To investigate the optical behaviour of AuNPs and AuNBPs, UV-visible spectroscopy studies  
193 were carried out in the 300 to 800 nm wavelength range. **Figure 2(a) curve i** depicts the  
194 absorption spectrum of a seed solution of AuNPs. Here, a single absorption peak appeared at  
195 529 nm due to SPR phenomenon in gold core [enlarge view shown in inset image] [19].

196 Time-dependency of AuNBPs' growth is a key feature to be analysed and estimated in order  
197 to fully control their synthesis. UV-visible spectra of AuNBPs solutions during growth were  
198 then acquired at 15 minutes, 1 hour, 15 hours and 24 hours. The corresponding results are  
199 shown in **Figure 2(a) ii to v**, respectively. In these spectra, two distinct plasmon resonance

200 peaks, at 550 nm and 700 nm, can be observed which can be assigned to longitudinal and  
201 transverse plasmon absorption, respectively [20]. The intensity of these peaks increases  
202 progressively with growth time indicating continuous formation and stabilization of AuNBPs.  
203 During the purification and ligand exchange steps, UV-visible studies were also carried out to  
204 monitor the colloidal stability of AuNBPs. **Figure 2(b)** presents the UV-visible absorption  
205 spectra of surface-modified AuNBPs before and after CTAB to lipoic acid exchange. As can  
206 be seen, the CTAB-AuNBPs' absorption peaks (539 nm and 686 nm) (**curve i**) remain  
207 unchanged after DCM washing (**curve ii**), demonstrating the stability and colloidal behaviour  
208 of DCM-washed AuNBPs. However, DCM-washed AuNBPs further treated with lipoic acid  
209 (**Figure 2(b) curve iii**) exhibited longitudinal and transverse peaks at 541 nm and 682 nm,  
210 respectively. Such a slight redshift shall be attributed to the electronic interaction between  
211 lipoic acid and AuNBPs [15].

212 The crystallinity and diffraction planes of AuNBPs were then investigated by X-ray  
213 diffraction technique [**Figure S1**]. The primary diffraction peak appeared at  $37.94^\circ$ ,  
214 corresponding to (111) diffraction planes. The other dominating peaks appeared at  $45.86^\circ$ ,  
215  $67.01^\circ$ ,  $76.40^\circ$  and were attributed to (200), (220), (311) planes, respectively. Four prominent  
216 peaks were then indexed as the cubic structure of gold, and no diffraction peaks were found  
217 for Pt structure, confirming the formation of pure Au nanostructures.

218 The transformation of shape and size from AuNPs to AuNBPs were confirmed by TEM  
219 microscopy. **Figure 3(a)** presents the TEM image of the seed solution of AuNPs. AuNPs  
220 were found to be monodisperse and characterised by an average 10 nm diameter. As-prepared  
221 AuNBPs (**Figure 3(b-f)**) exhibit bipyramid like morphology characterised by a geometry  
222 where the two pyramids share the same base. The average size of the AuNBPs was found to  
223 be 70 nm between the two apex and a maximum width of 35 nm along. Here, the use of the  
224 shape directing agent  $\text{H}_2\text{PtCl}_6$  in the presence of the ionic surfactant CTAB was shown to be  
225 highly efficient in controlling the final architecture of the bipyramids structure [14].

226 AFM studies of AuNBPs/SAM/ITO surface have been performed using Scanning Probe  
227 Microscope Solver PRO in contact force equipped with standard golden-silicon CSG01  
228 cantilevers having a 0.15 N/m spring constant. **Figure S2** describes the distribution of the  
229 nano-objects over the surface, characterised by a well-ordered and random arrangement of  
230 individual AuNBPs and the absence of aggregates.

231 Contact angle studies were carried to determine the hydrophilic or hydrophobic character of  
232 the modified electrode surfaces [**Figure S3 (a-c)**] using the sessile drop method. The contact  
233 angle of bare ITO electrode was found to be  $70.04^\circ$  [**Figure S3 (a)**], which decrease to  $29.68^\circ$



234 for AuNBPs/SAM/ITO electrode surface [**Figure S3 (b)**]. This decrease of the contact angle  
235 value was assigned to the introduction of hydrophilic groups through the presence of lipoic  
236 acid capped AuNBPs. After the immobilization of anti-AFB<sub>1</sub> on electrode surface [**Figure**  
237 **S3(c)**], the observed contact value was lowered to 10.31. This large decrease was a clear  
238 evidence of the grafting of hydrophilic proteins at the electrode surface [21].

239 **Figure 4 (a, b)** presents the FT-IR spectrum of CTAB-AuNBPs and lipoic acid-AuNBPs. In  
240 **Figure 4(a)**, the broad-spectrum peak observed at 3392 cm<sup>-1</sup> was identified as the stretching  
241 vibration of hydroxyl groups (OH). The strong absorptions occurring at 2945, and 2853 cm<sup>-1</sup>  
242 were attributed to CH symmetric and asymmetric vibration of methyl and methylene group of  
243 a long aliphatic chain of CTAB, respectively. The peaks observed at 1750, 1605 and 1466  
244 cm<sup>-1</sup> were attributed to C=O stretch of COOH groups and vibration of the aromatic ring. In  
245 the fingerprint region, the 1219 cm<sup>-1</sup> peak was attributed to C-O stretching vibration in  
246 CTAB-AuNBPs. The sharp peaks at 957 cm<sup>-1</sup> was identified as -C-N<sup>+</sup> stretching mode and  
247 the one at 711 cm<sup>-1</sup> as CTAB bond to Au surface. Exchange of capping ligand from CTAB to  
248 lipoic acid of AuNBPs was also confirmed by FT-IR technique. Indeed, **Figure 4(b)** presents  
249 the spectrum of lipoic acid capped AuNBPs, in which a peak appeared at 3464 cm<sup>-1</sup> which  
250 corresponds to OH groups in a carboxylic acid, specific here of the lipoic acid function. In the  
251 spectrum of lipoic acid-AuNBPs, a slight shift was also observed in the peak positions at  
252 3092 and 3001 cm<sup>-1</sup> due to aliphatic groups of lipoic acid. C-O and C=O bond peaks were  
253 also observed at 1637, and 1555 cm<sup>-1</sup>, indicating the electrostatically stabilized conjugation of  
254 lipoic acid with AuNBPs. 1249 and 1117 cm<sup>-1</sup> absorption bands were attributed to C=O  
255 stretching mode and in-plane C-H bending vibration, respectively. Finally, a peak appeared at  
256 764 cm<sup>-1</sup> which was assigned to bonding of lipoic acid with AuNBPs.

257 Surface coverage percentage as well as the distribution of anisotropic shape of AuNBPs over  
258 the ITO-coated glass electrode were studied through scanning electron microscopy imaging.  
259 **Figure S4 (a)** presents a close view of an AuNBPs/SAM/ITO surface. As can be seen,  
260 abundant anisotropic shape gold crystals covered the electrode surface and confirmed the  
261 conjugation of AuNBPs with the self-assembled ITO electrode.

262

### 263 **3.2 Optical characterization using the imaging SPR technique**

264 AuNBPs/SAM/ITO electrodes were used for imaging SPR studies. Scanning the angle of the  
265 incident light used for imaging SPR led us to demonstrate the presence of a large change of  
266 reflectivity at the surface-liquid interface of AuNBPs/SAM/ITO with a maximum value  
267 obtained at an angle of 56.73° [**Figure S5(a)**]. This first results suggested that the incident

268 light was matching with the surface plasmon but also experimentally demonstrated the  
269 phenomenal concept of LSPR at AuNBPs modified ITO glass substrate.

270 After confirming the presence of LSPR phenomenon based on AuNBPs/SAM/ITO surface,  
271 *in-situ* surface functionalization and modification were performed using SPR2 Horiba  
272 instrument and followed through imaging SPR measurements [**Figure S5 (b)**]. As can be  
273 seen, each step was easily followed and identified using imaging SPR variations.  
274 AuNBPs/SAM/ITO electrodes were first activated by injection of EDC-NHS (step 1) then the  
275 EDC-NHS solution was removed, washed with PBS buffer and replaced by 20  $\mu$ L of anti-  
276 AFB<sub>1</sub> antibodies solution at a concentration of 100  $\mu$ g/mL. A shift in reflectivity was clearly  
277 observed, indicating the binding of antibodies to the activated electrode surface (**step-2**) [22].  
278 Further, excess of antibodies was removed by washing the electrode with PBS, and the  
279 surface was passivated in the presence of an ethanolamine solution (**step-3**).

280

### 281 **3.3 Imaging SPR response towards AFB<sub>1</sub> target analyte**

282 Optical analytical performances of anti-AFB<sub>1</sub>/AuNBPs/ITO electrode were evaluated through  
283 the injection of various AFB<sub>1</sub> concentrations and quantification of the corresponding imaging  
284 SPR signals. **Figure 5(a)** presents the real time imaging SPR measurements obtained on anti-  
285 AFB<sub>1</sub>/AuNBPs/ITO electrode in the presence of AFB<sub>1</sub> in the 0.1-500 nM concentration  
286 range. Clear shifts in reflectivity were observed with increasing AFB<sub>1</sub> concentrations, due to  
287 the interaction between the immobilized antibodies and the injected AFB<sub>1</sub> [8]. From these  
288 experimental results, a dose-response curve was easily built which exhibits a semilog  
289 linearity ( $R^2=0.99$ ), compatible with the classical model of interaction (Langmuir model)  
290 [**Figure 5(b)**]. A limit of detection of 0.4 nM was calculated using the standard  
291 formula  $3\sigma/m$ , here  $\sigma$  is the standard deviation and  $m$  is the slope of the linearized calibration  
292 curve. The high sensitivity with lower limit of detection demonstrated that the fabricated  
293 electrode surface was potentially useful for AFB<sub>1</sub> detection. Electrode surfaces covered with  
294 AuNBPs were then proven to be powerful sensing tools with high sensitivity attributed to a  
295 LSPR-SPR coupling effect [23].

### 296 **3.4 Electrochemical characterization**

297 Electrochemical characterization studies of AuNBPs/SAM/ITO and anti-  
298 AFB<sub>1</sub>/AuNBPs/SAM/ITO were carried out through cyclic voltammetry and electrochemical  
299 impedance spectroscopy.

300 The Cyclic voltammetry studies of AuNBPs/SAM/ITO and anti-AFB<sub>1</sub>/AuNBPs/SAM/ITO  
 301 were performed in an electrolyte solution composed of 50 mM PBS (pH 7.4) containing in  
 302 addition 5 mM of [Fe(CN)<sub>6</sub>]<sup>3-/4-</sup> as a redox probe. The scanning was performed from -0.7 to  
 303 0.7 V vs Ag/AgCl reference at a 50 mV/s scan rate [**Figure S7 (a)**]. Cyclic voltammograms  
 304 of AuNBPs/SAM/ITO (**curve i**) electrodes exhibited well-defined oxidation and reduction  
 305 potential peaks at 0.24 V and 0.04 V, respectively, whereas anti-AFB<sub>1</sub>/AuNBPs/SAM/ITO  
 306 electrodes showed oxidation peak potential at 0.27 V and reduction peak potential at  
 307 0.0006V. These increase and decrease in oxidation and reduction peak potentials, were  
 308 attributed to high electrocatalytic activity and electrical conductivity of AuNBPs which  
 309 improve the electron transfer rate between electrode and the electrolyte redox probe [24].  
 310 Also, similar decrease in the current peak intensity was observed after the surface  
 311 functionalization with anti-AFB<sub>1</sub> antibodies on AuNBPs/SAM/ITO electrode. This change  
 312 was attributed to loading of antibodies over the nanomaterial matrix that creates hindrance in  
 313 transferring the electrons between electrode and electrolyte [25].

314 **Figure S6** presents the cyclic voltammetry scan rate study of anti-AFB<sub>1</sub>/AuNBPs/SAM/ITO  
 315 electrodes with scanning rate ranging from 10 to 100 mV/s. As can be seen, varying the scan  
 316 rate led to a linear variation of the anodic and cathodic peak currents as a function of the  
 317 square root of scan rate (**Figure S6 insert**). The linear increase in the current response  
 318 indicates a quasi-reversible diffusion-controlled process. The obtained linear anodic and  
 319 cathodic peak current response can be described by the following equations (1-2):

$$I_{ac} = 77.276 \mu A + 107.537(\mu A^2 mV^{-1} s)^{1/2} x [scan rate (mV s^{-1})]^{1/2}; R^2 = 0.9972 \quad (1)$$

$$I_{cc} = -169.51 \mu A - 71.9032(\mu A^2 mV^{-1} s)^{1/2} x [scan rate (mV s^{-1})]^{1/2}; R^2 = 0.9950 \quad (2)$$

320 Diffusion coefficient of modified electrodes AuNBPs/SAM/ITO and anti-  
 321 AFB<sub>1</sub>/AuNBPs/SAM/ITO was then calculated using Randles-Sevcik equation (3)

$$I_p = 2.69 x 10^5 n^3 A D^{1/2} V^{1/2} C \quad (3)$$

322 Here, I<sub>p</sub> is the peak current (μA), n is the number of electrons, C is the concentration of  
 323 electrolyte (5 mM), D is the diffusion coefficient, A is the area of the electrode and V is the  
 324 scan rate (50 mV/s). D value was found to be 9.375x10<sup>-6</sup> cm<sup>2</sup>/s and 8.01x10<sup>-6</sup> cm<sup>2</sup>/s for  
 325 AuNBPs/SAM/ITO and anti-AFB<sub>1</sub>/AuNBPs/SAM/ITO electrodes, respectively.

326 Following these cyclic voltammetry characterisations, electrochemical impedance  
 327 spectroscopy (EIS) was used to further characterise the obtained surfaces. **Figure S7 (b)**  
 328 illustrates the electrochemical impedance spectrum of AuNBPs/SAM/ITO and anti-  
 329 AFB<sub>1</sub>/AuNBPs/SAM/ITO. Measurements were here conducted in an electrolyte solution

330 composed of 50 mM PBS (pH 7.4) containing in addition 5 mM of  $[\text{Fe}(\text{CN})_6]^{3-/4-}$ . The charge  
331 transfer resistance ( $R_{\text{CT}}$ ) value of AuNBPs/SAM/ITO film was found to be 265.39  $\Omega$  and was  
332 attributed to the electronic conductivity of AuNBPs. After the immobilization of anti-AFB<sub>1</sub>  
333 on the AuNBPs/SAM/ITO electrode,  $R_{\text{CT}}$  value was found to have increased to 533.82  $\Omega$ .  
334 These results confirmed that a large amount of antibodies has been grafted on the electrode  
335 surface, leading to steric hindrance in transferring the electron between the electrode surface  
336 and the electrolyte solution.

337 Another important parameter in electrochemistry is the heterogeneous electron transfer  $K_e$   
338 which can be calculated by equation 4 [26]

$$K_e = \frac{RT}{n^2 F^2 A R_{\text{CT}} C} \quad (4)$$

339 Here, R is the gas constant (8.314 J mol<sup>-1</sup> k<sup>-1</sup>), T is the temperature (298 k), n is the number of  
340 electrons (n=1), A is the area of electrode (cm<sup>2</sup>), C is the concentration of electrolyte (5mM),  
341 F is the Faraday constant (96500 cm mol<sup>-1</sup>) and  $R_{\text{CT}}$  is the charge transfer resistance ( $\Omega$ ). The  
342  $K_e$  value of AuNBPs/SAM/ITO electrode was found to be  $4.0 \times 10^5$  cm s<sup>-1</sup>. This value was  
343 found to decrease to  $1.9 \times 10^5$  cm s<sup>-1</sup> after the immobilization of anti-AFB<sub>1</sub> on  
344 AuNBPs/SAM/ITO electrode due to heavier bulky group present in the structure of antibody  
345 which creates steric hindrance in transferring the electron from electrode to the electrolyte  
346 solution.

### 347 **3.5 EIS response studies of biosensor towards AFB<sub>1</sub> target analyte**

348 In order to investigate the electrochemical response of anti-AFB<sub>1</sub>/AuNBPs/SAM/ITO  
349 electrodes, EIS measurements were carried out in the presence of various AFB<sub>1</sub>  
350 concentrations (**Figure 6(a)**). As can be seen, increasing the concentration of AFB<sub>1</sub>, from 0.1  
351 to 25 nM, led to the increasing of the characteristic semi-circle diameters of the obtained  
352 Nyquist plots. This behaviour was expected and is related to the binding of antigen to binding  
353 sites of antibodies. The formation of this immunocomplex at the electrode surface greatly  
354 lower the electron transfer rate between the modified surface and  $[\text{Fe}(\text{CN})_6]^{3-/4-}$  redox probe.  
355 It is worth to mention that for AFB<sub>1</sub> concentrations higher than 25 nM, no more change in the  
356 impedance signal was observed, thus indicating that all binding sites of the modified  
357 electrodes were already occupied. **Figure 6(b)** presents the calibration curves plotted using  
358 the  $R_{\text{CT}}$  values calculated for each AFB<sub>1</sub> concentration. Once again, a linear correlation of the  
359 semilog curve was found with a linear regression coefficient ( $R^2$ ) value of 0.9824, and a

360 standard deviation of 0.879. The limit of detection (LOD), calculated as before using the  
361 standard formula  $3\sigma/m$ , was found to be 0.1 nM. Thus, the AuNBPs based electrochemical  
362 immunosensor exhibit high sensitivity with a lower limit of detection. In comparison, Sharma  
363 et al. have developed cystamine functionalized gold nanoparticles integrated in an  
364 electrochemical immunosensor for AFB<sub>1</sub> detection [27]. These fabricated immune-electrodes  
365 were responding in the range of 10-100 ng dL<sup>-1</sup> with a LOD of 17.90 ng dL<sup>-1</sup> and sensitivity  
366 of 0.45 μA ng<sup>-1</sup> dL. Also, Yagati et al. have constructed AFB<sub>1</sub> immuno-electrode using Au-  
367 PANI composite as sensor exhibiting linear range of detection from 0.1 to 100 ng/mL [28].  
368 The use of Au-PANI material showed effective contribution for fabricating the sensing  
369 platform due to high surface area, improved electrochemical conductivity and its  
370 biocompatibility properties.

### 371 **3.6 Analysis in maize sample using the EIS technique**

372 Fresh maize samples were used to investigate the analytical performances of the anti-  
373 AFB<sub>1</sub>/AuNBPs/ITO surfaces using the EIS technique. Different concentrations of AFB<sub>1</sub> were  
374 spiked in maize samples, extracted and quantified using the **Figure 6(b)** calibration curve.  
375 The obtained recovery results are presented in **Supplementary-Table 1**. As a matter of fact,  
376 the obtained recovery of percentages were all higher than 95%, indicating that the developed  
377 biosensor had the capability to detect its target in a complex matrix such as maize.

378

### 379 **4. Conclusions**

380 Nanostructures of AuNPs and AuNBPs were successfully synthesized using a seed-mediated  
381 growth process and time-dependent growth studies were investigated using UV-visible  
382 spectroscopy. In order to exchange the capping agent of AuNBPs from surfactant to lipoic  
383 acid, ligand exchange process was developed for covalent attachment with minimal steric  
384 hindrance on amine-functionalized self-assembled monolayer ITO-glass surface. Shape and  
385 size of the AuNBPs were confirmed by TEM and revealed average length size of 70 nm and  
386 width size of 35 nm for bipyramid sharing the same base. **Imaging SPR biosensing response**  
387 **of anti-AFB<sub>1</sub>/AuNBPs/SAM/ITO surfaces shown gradual increment in the reflectivity signal**  
388 **as by increasing the AFB<sub>1</sub> concentration in the range of 0.1 to 500 nM with a LOD of 0.4 nM.**  
389 **Sequentially, electrochemical biosensing studies were carried out using the EIS technique and**  
390 **demonstrated that impedimetric response ( $R_{CT}$ ) increased with increasing concentration of**  
391 **AFB<sub>1</sub> from 0.1 to 25 nM with a LOD of 0.1 nM.** Fabricated electrochemically biosensor was

392 tested to analyse spiked maize samples and was shown to lead to acceptable percentage of the  
393 recovery.

394

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