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Highly-efficient electrochemical label-free immunosensor for the detection of ochratoxin A in coffee samples

Kwanele Kunene^{a,b}, Matthieu Weber^a, Myalowenkosi Sabela^b, Damien Voiry^a, Suvadhan Kanchi^b, Krishna Bisetty^{b,*} and Mikhael Bechelany^{a,*}

^a*Institut Européen des Membranes, UMR 5635 ENSCM-UM-CNRS Université Montpellier, Place Eugene Bataillon, F-34095 Montpellier Cedex 5, France*

^b*Department of Chemistry, Durban University of Technology, P.O. Box 1334, Durban 4000, South Africa*

†Corresponding authors. mikhael.bechelany@umontpellier.fr (M. Bechelany), bisettyk@dut.ac.za (K. Bisetty)

Abstract

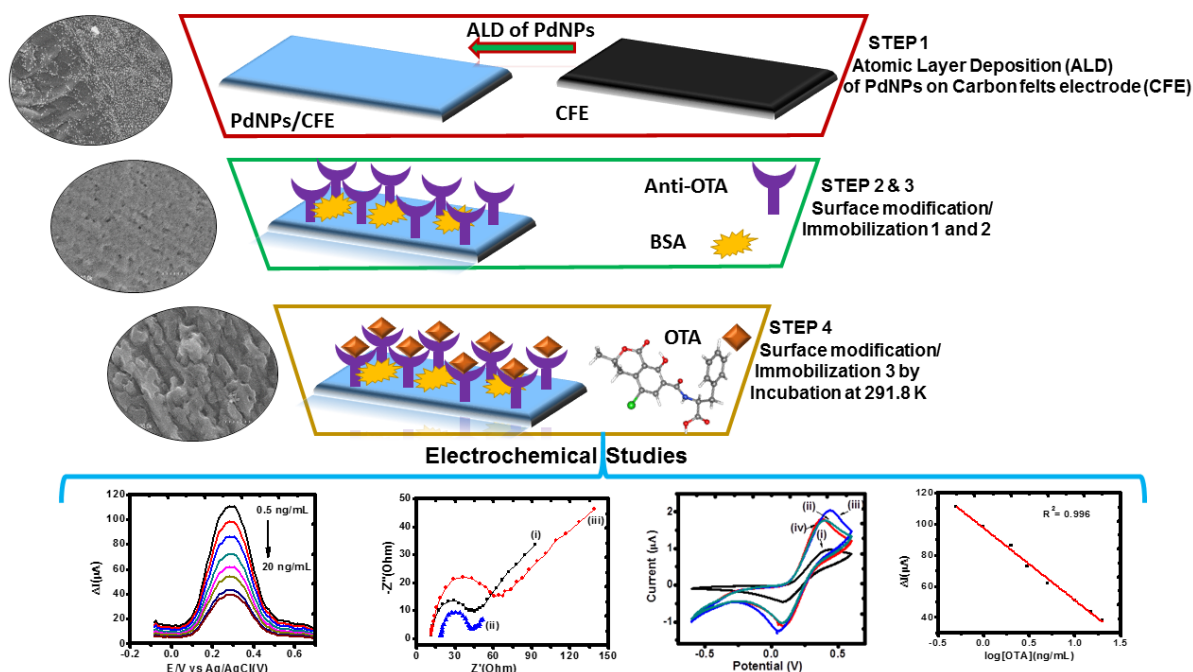
Ochratoxin A (OTA) is among the most important mycotoxins classified as potential risks to human health and food safety. In this work, a novel label-free electrochemical immunosensor has been proposed for the quantitative detection of OTA, based on a two-step strategy for the fabrication of the immunosensor. This involved coating of a carbon felt (CF) electrode with palladium nanoparticles (PdNPs) using atomic layer deposition (ALD), followed by the grafting of the anti-OTA antibodies onto the nanocomposite structure using a carbodiimide functional group via a cross linkage route. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) have been employed for the characterization of the immunosensor properties. The fabricated BSA/anti-OTA/PdNPs/CF immunosensor showed outstanding electrochemical performance towards the detection of OTA in spiked coffee samples. At the optimal working conditions, the linear detection range of the developed immunosensor was from 0.5-20 ng mL⁻¹ ($R^2 = 0.996$) with a low detection limit of 0.096 ng mL⁻¹, making it applicable to the screening of OTA in food products. In addition, the sensor was highly selective to OTA in the presence of interfering compounds and revealed stability

28 of up to three weeks, opening up prospects for the molecular sensing community and paving a
29 new route for quality control in the food industry.

30

31 **Keywords:** Electrochemical immunosensor, Ochratoxin A, Carbon felts electrode, Palladium
32 nanoparticles, Atomic Layer Deposition

33



34

35

36 1. Introduction

37

38 Ochratoxin A (OTA) is one of the secondary fungal metabolite that occurs naturally and is
39 present in many food products and produced by a number of fungal species such as
40 *Aspergillus ochraceus* and *Penicillium verrucosum* [1]. OTA is primarily found in food
41 commodities such as cereals, coffee beans, and wine [2-4]. Different studies reported that
42 OTA is among the most abundant and toxic mycotoxins due to their high hepatotoxic,
43 nephrotoxic, teratogenic, and mutagenic effects to most mammalian species [5, 6]. They are
44 suspected of being one of the main cause of immuno suppression and immuno toxicity [7],

45 therefore, policies have been implemented in order to limit their toxicity in food products. For
46 example, the European Union (E.U.) has established maximum permitted limits for OTA
47 depending on the food products: 5.0 $\mu\text{g kg}^{-1}$ for unprocessed cereals, 10.0 $\mu\text{g kg}^{-1}$ for dried
48 fruits, 15.0 $\mu\text{g kg}^{-1}$ for spices, 2.0 mg mL^{-1} for all types of wines and 10 ng mL^{-1} for coffee
49 beans [2, 8, 9]. Consequently, the development of suitable analytical techniques to efficiently
50 monitor OTA concentrations in food products has become crucial.

51 Conventional analytical techniques for the determination of OTA involves thin layer
52 chromatography (TLC) [10], gas chromatography (GC) [11], liquid chromatography mass
53 spectrometry (LC/MS) [12], photoluminescence [13, 14], enzyme-linked immunosorbent
54 assay (ELISA) [15] ultra high performance chromatography-tandem mass spectrometry
55 (UHPLC-MS/MS) [16] and high performance liquid chromatography (HPLC) [17, 18].
56 However, these techniques require extensive sample preparation, highly trained personnel and
57 are time consuming and costly. Thus, alternative methods offering high sensitivity, cost-
58 effectiveness, fast and portable detection such as fluorescence [19], chemiluminescence [20]
59 or electrochemistry [21, 22] have been developed. Previous reports have shown that
60 electrochemistry techniques are fast and sensitive (in the ng mL^{-1} range), but with limited
61 selectivity. For this reason, a steady shift towards aptasensors or biosensor techniques has
62 been implemented, opening new promising paths for analysts. [19, 23].

63 Previously, various nanomaterials have been used for the development of efficient
64 electrochemical immunosensors for OTA detection in different food products [24, 25]. As
65 reported by Taghdisi and co-workers [26], the use of nanoparticles in electrochemical sensing
66 devices is an extremely promising prospect, since they are biocompatible and able to retain
67 the biological activity upon absorption, to enable the direct electron transfer through the
68 conducting tunnels and the enhancement of immobilization of antibodies [27, 28]. Bonel and

69 co-workers developed indirect and competitive electrochemical immunosensors for the
70 detection of OTA in wheat, using screen-printed carbon electrode (SPCE) on which OTA
71 were conjugated to bovine serum albumin (OTA-BSA) and gold nanoparticles (OTA-BSA-
72 AuNPs). The immunosensor showed a linear detection range (LDR) of 0.3 to 8.5 ng mL⁻¹,
73 with a limit of detection (LOD) of 0.86 ng mL⁻¹ [25]. Another biosensor was reported by
74 Rivas and co-workers where (SPCE) modified with polythionine (PTH) and iridium oxide
75 nanoparticles (IrO₂ NPs) were used for the detection of OTA in wine samples. The label-free
76 aptasensor showed the LDR of 0.004 and 40 ng mL⁻¹, and found the lowest LOD reported so
77 far for label-free impedimetric detection of OTA (14 pM) [24]. Karczmarczyk and co-workers
78 developed a sensitive indirect competitive assay quartz crystal microbalance with dissipation
79 monitoring (QCM-D) sensor for detection of OTA in red wine. They amplified the QCM-D
80 signal by combining the secondary antibodies with gold nanoparticles (AuNPs), and found a
81 LODs of 0.16 ng mL⁻¹ in the LDR of 0.2–40 ng mL⁻¹ [4]. Zhang and co-workers represents
82 the amplified voltammetric immunoassay for OTA in red wine. They achieved this by
83 enclosing platinum on gold cores (AuPtNP) and functionalized it with monoclonal antibodies.
84 The system presented a LDR of 0.2 to 5×10³ pg mL⁻¹ of OTA, with a lower LOD of 0.75 pg
85 mL⁻¹ [29]. However, the above reported immunosensors for OTA detection were based on
86 complex methods for synthesis of nanomaterials and were often fabricated using multiple
87 routes. Thus, novel and easier routes enabling the fabrication of immunosensing devices are
88 highly desired.

89 Atomic layer deposition (ALD) is a novel promising strategy for the direct growth of both
90 thin films and nanoparticles with controllable dimensions at the nanometer scale [30, 31].
91 This vapor phase technology is based on the sequential use of self-limiting chemical reactions,
92 enabling the synthesis of high quality inorganic nanomaterials in challenging substrates, with

93 a precise control over their thickness. These benefits permitted this technique to become an
94 essential tool for the deposition of nanomaterials for a myriad of applications, such as
95 microelectronics [32], but also catalysis [33], membranes [34] and biosensing [35].

96 In the present work, we report a novel PdNPs/carbon nanocomposite based immunosensor for
97 the efficient detection of OTA in coffee samples, using a two-step strategy for the fabrication
98 of the immunosensor. Firstly, ALD was employed to coat CF substrates with highly dispersed
99 palladium nanoparticles (PdNPs), and the anti-OTA were then grafted to the composite
100 structure using a carbodiimide cross linkage route. Next, the composite structures were
101 characterized in terms of physical and chemical properties, using scanning electron
102 microscopy (SEM), transmission electron microscopy (TEM) and attenuated total reflectance
103 (ATR). The immunosensor was then tested for the detection of OTA in spiked coffee samples
104 using differential pulse voltammetry (DPV) and assessed on LODs, reproducibility and
105 selectivity.

106

107 **2. Experimental section**

108 **2.1 Materials and methods**

109 The carbon felt (CF) AvCarb® MGL190 was purchased from Johnson Matthey Co.,
110 Germany. Modified carbon felt electrode (CFE) were used as the working electrode, graphite
111 rode as the counter electrode and Ag/AgCl as the reference electrode. The dimensions of the
112 working electrode supports were 3.5 cm length, 0.7 cm width, and 0.3 cm thickness. *N*-(3-
113 dimethylaminopropyl)-*N*'-ethycabordiimide (EDC)(CAS Number 1892-57-5, purity 97.0%),
114 *N*-hydroxysuccinimide (NHS)(CAS Number 6066-82-6, purity 98.0%), potassium
115 ferricyanide ($K_3Fe(CN)_6$) (CAS Number 13746-66-2, purity 99.0%), potassium ferrocyanide
116 ($K_4Fe(CN)_6$) (CAS Number 14459-95-1, purity 98.5-102.0%), potassium hydrogen phosphate

117 (K₂HPO₄) (CAS Number 7758-11-4, purity 98.0%), potassium dihydrogen phosphate
118 (KH₂PO₄) (CAS Number 7778-77-0, purity 99.0%), phosphate buffered saline tablet (PBS)
119 (CAS Number 0000000000), palladium (II) hexafluoroacetylacetonate (Pd(hfac)₂ (CAS
120 Number 64916-48-9, purity 99.0%), OTA standard solution (OTA), 1 mg mL⁻¹ in DMSO
121 (CAS Number 303-47-9, purity 98.0%), formaldehyde solution (CH₂O) (CAS Number 50-00-
122 0, ACS reagent, 37 wt. % in H₂O, contains 10-15% Methanol as stabilizer) and Bovine serum
123 albumin solution (BSA) (CAS Number 9048-46-8, purity 98.0%) were purchased from
124 Sigma-Aldrich, France. Monoclonal antibody anti-ochratoxin A (anti-OTA) (Catalog #:
125 ICP9948, 250 μg mL⁻¹ in PBS 50% glycerol) was obtained from Immune Chem
126 Pharmaceutical Incl (Canada). Nescafe (NES, Vevey, Switzerland) obtained from a local
127 supermarket. Double distilled water was used for all experiments.

128

129 **2.2 Apparatus**

130 The modified CF electrodes were characterized by scanning electron microscopy
131 (SEM, Hitachi S-4800). The contact angle (CA) measurements of PdNPs/CF and BSA/anti-
132 OTA/PdNPs/CF electrodes were conducted on a homemade contact angle setup. During
133 measurement, a drop of deionized water was deposited over the electrode surface and the
134 angle of the liquid surface with contact surface was observed at the solid-liquid interface.
135 attenuated total reflectance (ATR) spectra were collected using iS50 ATR Thermo scientific
136 spectrophotometer. Electrochemical measurements such as cyclic voltammetry (CV),
137 differential pulsed voltammetry (DPV) and electrochemical impedance spectroscopy (EIS)
138 were performed at room temperature (~ 25.0 °C) using a SP-150 EC-LAB Electrochemistry
139 chemical workstation (VSP Potentiostat from BioLogic Science Instruments, France).
140 Transmission electron microscopy (TEM) was performed using a JEOL 2200FS (200 kV).

141

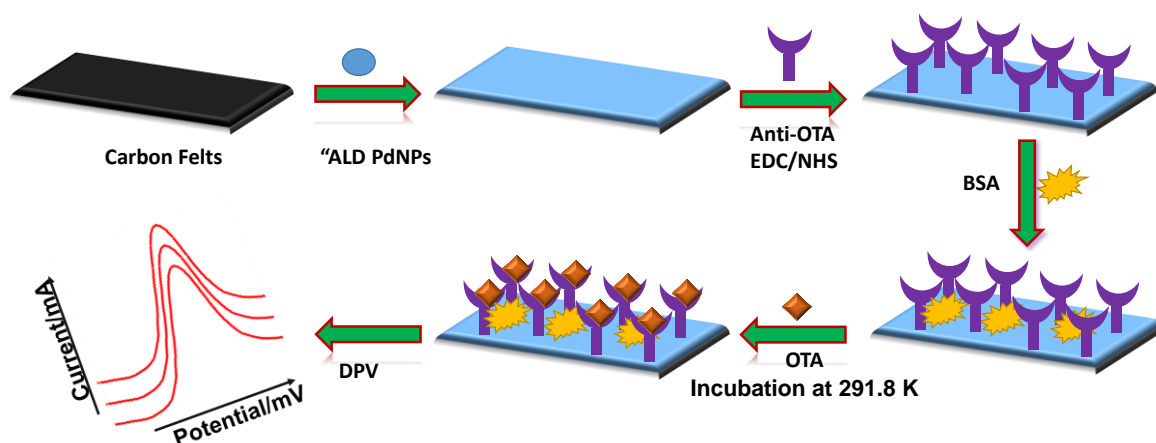
142 **2.3 Synthesis of palladium nanoparticles (PdNPs) by Atomic Layer Deposition (ALD)**

143 In this work, the highly dispersed PdNPs were synthesized by applying 200 ALD
144 cycles in a low-pressure hot-wall (home-built) ALD reactor. ALD was achieved using
145 sequential exposures of Pd(hfac)₂ and formalin separated by Argon purges. If not specified
146 otherwise, the ALD cycle consisted of 5 s pulse of Pd(hfac)₂, 15 s of gas exposure, 10 s of
147 purge with Argon followed by 1 s pulse of formalin, 15 s of exposure and finally 60 s purge
148 with Argon. Further details about both this deposition protocol and the associated ALD
149 reactor can be found elsewhere [35, 36].

150

151 **2.4 Modification of PdNPs/CF with anti-OTA and BSA**

152 A fresh stock solution of anti-OTA (1.0 µg mL⁻¹) was prepared in phosphate buffer
153 saline solution (PBS) presenting a pH value of 7.4. The anti-OTA solution was mixed with
154 0.4 M EDC and 0.1 M NHS in the ratio of 4:1:1 and kept at 4.0 °C for 30 min, to activate the
155 carboxyl groups in fragment crystallizable (Fc) region of anti-OTA [37]. Thereafter, the anti-
156 OTA was ready for the two steps immobilization process onto the surface of PdNPs/CF. In a
157 first step, 10 µL of anti-OTA with EDC-NHS was spread over the PdNPs/CF electrode and
158 incubated at 4.0 °C for 6.0 h, after which it was washed with PBS to remove the unbounded
159 or excess anti-OTA from the electrode surface. Secondly, 10 µL of BSA (0.1 %) was spread
160 over anti-OTA/PdNPs/CF immunoelectrode surface, to block any non-specific active sites on
161 the electrode (Scheme 1). The fabricated BSA/anti-OTA/PdNPs/CF immunoelectrode was
162 kept at 4.0 °C when not in use.



163

164 **Scheme 1:** Schematic representation of the preparation of BSA/anti-OTA/PdNPs/CF
 165 immunoelectrode.

166

167 2.5 Preparation of coffee samples

168 The stock solution of the coffee sample (1.0 mg mL^{-1}) was prepared by ultrasonically
 169 mixing 10 mL of PBS and 10 mg of coffee for 2 h. Thereafter 1.0 mL of the prepared
 170 stock solution was spiked with different concentrations of OTA ranging from 0.5 to 20 ng
 171 mL^{-1}) and kept at $4.0 \text{ }^\circ\text{C}$ until further use.

172

173 2.6 Indirect detection of OTA

174 For the OTA measurements, $10 \text{ } \mu\text{L}$ of OTA standards with different concentrations
 175 ranging from 0.5 to 20 ng mL^{-1} in PBS was pipetted onto the surface of the BSA/anti-
 176 OTA/PdNPs/CF immunoelectrodes and allowed to stand for 40 min at room temperature.
 177 DPV was used for the quantification of OTA and the measurements were conducted using a

178 5.0 mM $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (1:1) mixture in PBS (pH 7.0). The CVs were performed in
179 5.0 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ supported by 1 M KCl in 0.1 M $\text{K}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$ solution. The EIS
180 measurement was performed in 1 M KCl containing equimolar $[\text{Fe}(\text{CN})_6]^{3-/4-}$ with AC
181 frequency from 0.1 to 10^5 Hz.

182

183 3. Results and Discussion

184

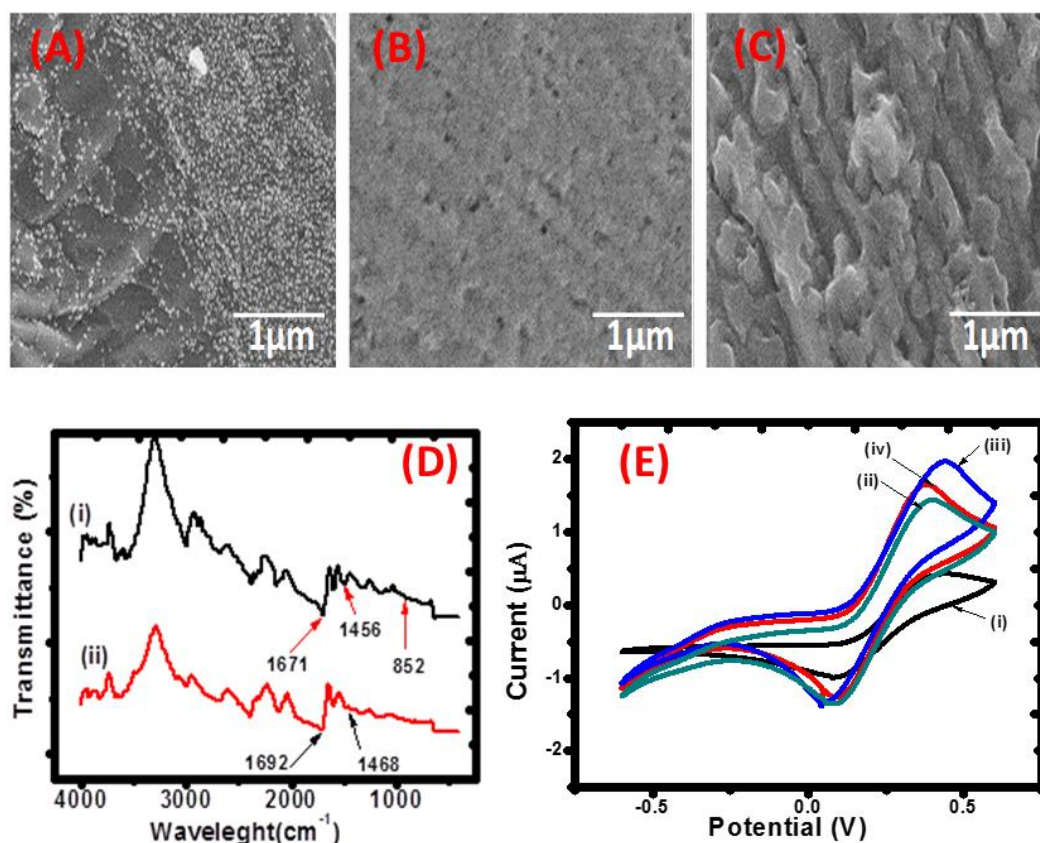
185 3.1. Physical and chemical characterizations of the electrodes

186 Surface morphology of the fabricated electrodes were characterized by SEM and TEM.
187 Fig 1A shows the morphology of PdNPs deposited onto CF by ALD over 200 cycles resulting
188 in the formation of uniformly dispersed NPs. The TEM images (Fig.S1 A, B & C) further
189 revealed an average diameter of 6 ± 2 nm for the PdNPs layer on the carbon substrate. It is
190 clear from the SEM and the TEM images that the PdNPs were well-dispersed at the surface of
191 the carbon substrate. These results were supported with X-Ray photoelectron microscopy to
192 confirm the pure metallic form of Pd and inductively coupled plasma mass spectrometry
193 (ICP-MS) to confirm the low metal loading limited at 0.85 wt.% (± 0.1 %), corresponding to
194 $<0.1 \text{ mgPd cm}^{-2}$ [35].

195 After immobilization of BSA onto anti-OTA/PdNPs/CF electrode, a smooth surface
196 morphology was obtained as shown in Fig. 1B. The BSA was used to block non-binding sites
197 of the anti-OTA/PdNPs/CF immunoelectrode. Further immobilization of OTA by incubation
198 onto BSA/anti-OTA/PdNPs/CF results in a rough surface as can be seen in Fig.1C, which is
199 indicative of optimum adsorption of OTA on the electrode surface.

200 The functional groups present in the fabricated immunosensor were investigated by ATR,
201 with Fig. 1D showing the anti-OTA/PdNPs/CF (curve i) and BSA/anti-OTA/PdNPs/CF

202 (curve ii). A characteristic peak at 1671 cm^{-1} corresponding to -NH deformation in an amide-
 203 II bond, suggesting the covalent immobilization of anti-OTA on the electrode surface (curve
 204 i). The band seen around 1456 cm^{-1} is due to the vibration of -CH₂ aliphatic moiety of anti-
 205 OTA [37]. The band found at 852 cm^{-1} is due to free -NH₂ groups on the electrode surface.
 206 After BSA immobilization (curve ii; BSA/anti-OTA/PdNPs/CF), the band at 852 cm^{-1}
 207 completely disappeared, this confirms the blocking of nonspecific sites available on anti-
 208 OTA/PdNPs/CF immunoelectrode [38].



209
 210 **Fig.1.** SEM images of PdNPs/CF electrode (A); BSA/anti-OTA/PdNPs/CF (B); and OTA/BSA/anti-
 211 OTA/PdNPs/CF (C) immunoelectrode. (D) ATR spectra of anti-OTA/PdNPs/CF (i); and BSA/ant-
 212 OTA/PdNPs/CF (ii) immunoelectrode; (E) CV comparison of CF electrode (i); PdNPs/CF (ii); anti-
 213 OTA/PdNPs/CF (iii) and BSA/anti-OTA/PdNPs/CF (iv) immunoelectrodes in PBS containing 5.0 mM
 214 [Fe(CN)₆]^{3-/4-} solution.

215

216 The hydrophobic/hydrophilic nature of the modified electrode was investigated by
217 measuring the water contact angle (CA) of the PdNPs/CF and BSA/anti-OTA/PdNPs/CF
218 electrodes. The CA represents the level of wetting property on the solid-liquid interaction. A
219 CA value of 56.2° for PdNPs electrode (Fig. S2A) indicates a reasonable hydrophilicity
220 however, after immobilization of anti-OTA onto the PdNPs/CF electrode (Fig S2B) the CA
221 value decreased to 14.3°, indicating that anti-OTA further enhanced the wettability properties
222 of the electrode.

223

224 **3.2. Electrochemical studies**

225 **3.2.1. Electrodes characterization through cyclic voltammetry (CV)**

226 CV is one of the most convenient technique that is used to monitor the behavior of the
227 modified electrode. Fig. 1 (E) shows the CV response obtained using 5.0 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in
228 PBS for (i) CF (ii), PdNPs/CF (iii) anti-OTA/PdNPs/CF and (iv) BSA/anti-OTA/PdNPs/CF
229 immunoelectrodes. A pair of well-defined redox peak was observed for the CF (curve i), This
230 quasi-reversible redox peak was attributed to the transformation between $\text{Fe}(\text{CN})_6^{4-}$ and
231 $\text{Fe}(\text{CN})_6^{3-}$. The low anodic peak current (I_{pa}) of 0.99 μA and 1.03 μA for the bare CF
232 electrode and anti-OTA/CFE immunoelectrode demonstrates a poor electrochemical response
233 of the CF electrode and hindrance of electron transfer caused by the insulation and steric
234 hindrance produced by anti-OTA. On the other hand, the I_{pa} increased to 1.77 μA for the
235 PdNPs/CF coated surface (curve ii). These results demonstrate that deposition of PdNPs onto
236 the CF substrate accelerates the rate of electron transfer between analyte and working
237 electrode, due to high surface area and improvement in catalytic activity of the electrode.

238 However, when anti-OTA were immobilized onto the PdNPs/CF electrode the I_{pa} increased to
239 2.05 μA (curve iii), indicating further enhanced sensitivity. This phenomenon is probably due
240 to the fragmented crystalline (Fc) region of the anti-OTA and the amine groups that forms a
241 penetrating path between anti-OTA and electrode [37]. The free site amino group of anti-OTA
242 available onto immunoelectrode surface electrostatically interacts with redox species of
243 electrolyte and facilitates the fast electron diffusion at the electrode. However, for the
244 BSA/anti-OTA/PdNPs/CF electrode the I_{pa} decreased to 1.83 μA (curve iv), this is in
245 agreement with the previous report stating that BSA inhibiting the diffusion of redox species
246 towards the electrode [39]. Our results confirmed the successful fabrication of the BSA/anti-
247 OTA/PdNPs/CF immunoelectrode.

248

249 3.2.2. Effects of scan rate

250 Cyclic voltammetry was used to study the interface kinetics of BSA/anti-
251 OTA/PdNPs/CF immunoelectrode by varying scan rate from 10-100 mV/s as shown in Fig.
252 S3A. The peak currents increase linearly with the increase of scan rates while there was a
253 minor shift of peak potential towards more a positive potential and more faradic current is
254 flowing on the electrode. This indicates that the electroactive species are confined at the
255 electrode surface and the reaction of OTA is following an adsorption-controlled process [40].

256

257 3.2.3. Electrochemical impedance spectroscopy

258 Electrochemical impedance spectroscopy results are presented using a Nyquist plot of CF
259 with different modification processes using $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as the electrolyte. EIS spectrum
260 comprises of a semicircle and the linear part as illustrated in Figures 2A-B. The semicircle
261 diameter represents the electron-transfer resistance (R_{ct}) and reveals the restricted diffusion of

262 the electrolyte through the multilayer system, directly related to the film permeability. A very
263 small semicircle diameter is observed on anti-OTA/CF electrode Fig. S6A demonstrating a
264 low charge transfer resistance for the electrochemical process. At low frequency, the linear
265 part is associated with the mass transfer process. After the deposition of PdNPs on the CF, the
266 capacitance increases. Fig. 2B (curve i) shows the modification with PdNPs increases the
267 electrochemical active surface area. The amplification of electrochemical signal and the
268 enhancement of the electron transfer rate of the sensor are due to the excellent electro-
269 catalytic activity of PdNPs [41]. After immobilization of anti-OTA (curve ii) onto PdNPs/CF
270 electrode, a remarkable decrease of the charge transfers resistance (R_{ct}) is observed. This
271 phenomenon is attributed to the presence of positively charged amino residues on the
272 antibody structure, which facilitates the electrochemical reaction [42]. The increased in
273 electron transfer observed can also be attributed to the neutralization of surface negative
274 charge upon reaction with EDC/Sulfo-NHS [43].

275 However, after immobilization of BSA (curve (iii)), both R_{ct} and the capacitance increased,
276 due to the longer path for the electrons to move from the solution to the surface of the
277 electrode. The EIS data in Fig. 2 (A and B) were further analyzed by fitting them to the
278 simulation data using the equivalent circuit model shown in Fig. 2 (B) inset. The fitting
279 parameters include the ohmic resistance of the electrolyte solution (R_s), C is the capacitance
280 that arises due to coverage of the electrode surface with BSA, R_{ct} is a charge transfer
281 resistance that is caused by the resistance of electrons between electrode and $Fe(CN)_6^{3-/4-}$
282 redox probe, R is electrolyte resistance in the pore and Q is the CPE arising due to CF surface
283 and Warburg impedance (W). Yang and co-workers reported the similar equivalent circuit on
284 their work, their equivalent fitting has, the interface ohmic resistance (R_d), double layer
285 capacitances (CPE_{dl}) and pore adsorption capacitance (CPE_{ad}) [44]. Siddiqui also reported the

286 similar equivalent circuit that has the uncompensated resistance R_s , capacitance C , charge
287 transfer resistance R_{ct} , R is electrolyte resistance in the pore and Q [45]. BSA layer makes the
288 electrode surface more homogenous and generates the capacitance of 0.4×10^{-8} F. Therefore,
289 BSA behaves as an insulator. Moreover, the ohmic resistance (R_s) of BSA/anti-
290 OTA/PdNPs/CF is estimated to be ~ 8.06 ohms, much lower than that of CF materials (~ 19.22
291 ohms) and PdNPs (~ 10.7 ohms).

292

293 **3.2.4. Effect of pH**

294 The pH is a key parameter when fabricating an immunosensor electrode, due to the strong
295 influence of the electrolyte on the electrochemical performance. This parameter was
296 investigated by monitoring the current response of immunoelectrodes in electrolytes in the
297 range of pH 6.0 - 8.0. In Fig. 2C the peak currents response increase from pH 6.0 to 7.0, then
298 gradually decreases beyond pH 7.0. This indicates that biomolecules on the electrode surface
299 can only provide optimum performance when they are on their original form at neutral pH as
300 the basic or acidic medium denatures them due to the interaction of H^+ or OH^- ion with amino
301 acid sequence of antibodies (anti-OTA) [39, 46]. The maximum value of the peak current was
302 observed at pH 7.0 and therefore it was selected as the optimum pH for the subsequent
303 experiments.

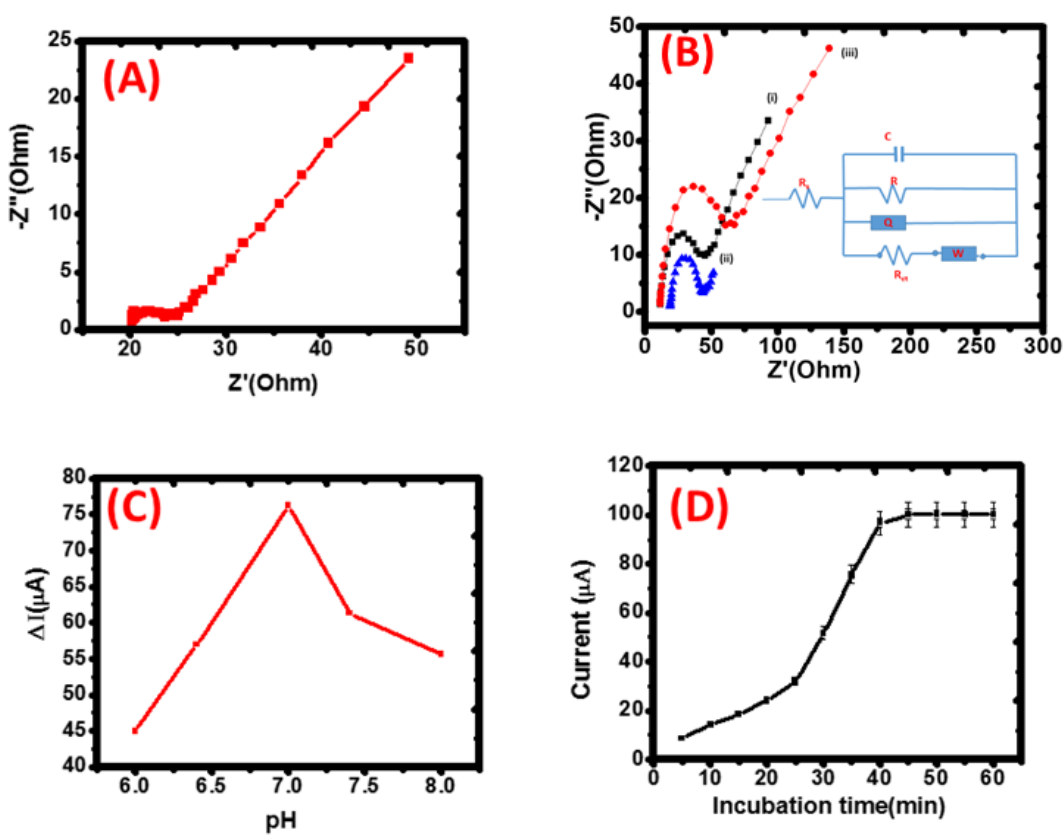
304

305 **3.2.5. Effect of incubation time**

306 The immunochemical reaction is the process whereby the antigen and antibody interacts
307 with each other to form the immunocomplex. Its formation depends on the interaction time
308 (incubation time) of the antibody and antigen. Therefore, in order to get the optimum value of

309 the incubation time, measurements of 1.0 ng mL^{-1} OTA on BSA/anti-OTA/PdNPs/CF
 310 immunoelectrode were recorded every 5 min for a duration of 60 min as shown in Fig. 2D. It
 311 was observed that the peak current rises with an increase in interaction time of the
 312 immunocomplex up to 40 min. Beyond 40 min, it remains constants due to the saturation of
 313 antibodies. Subsequently, duration of 40 min was selected as the optimum interaction time for
 314 the immunochemical interaction.

315



316

317

318 **Fig. 2** (A) Nyquist plots of bare CF electrode; (B) PdNPs/CF (i); anti-OTA/PdNPs/CF (ii)
 319 and BSA/anti-OTA/PdNPs/CF (iii) modified electrode in PBS, pH 7.0., containing 5.0
 320 mM $[\text{Fe}(\text{CN})_6]^{4-/3-}$ solution: the inset shows the used equivalent circuit. (C) DPV response
 321 of BSA/anti-OTA/PdNPs/CF immunoelectrode of electrolyte pH and (D) incubation time.

322

323

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327

328 3.2.6. Effects of anti-OTA concentration

329 The sensitivity of the immunosensor depends on the immunochemical reaction between
330 the antigen and antibody (anti-OTA). The concentration of the antibody on the electrode
331 surface is one of the most vital factors for the performance of the immunosensor. The effects
332 of anti-OTA concentration on the immunoelectrode were investigated by immobilizing four
333 different concentrations (0.5, 1, 5 and 10 $\mu\text{g mL}^{-1}$) of anti-OTA onto the PdNPs/CF electrode.
334 The DPV responses of OTA were measured from 0.5 ng mL^{-1} to 2.5 ng mL^{-1} , in order to
335 check the sensitivity of the fabricated immunoelectrodes. The change in current (denoted as
336 ΔC), measured before and after immunoreaction, was calculated according to equation (1):

$$337 \quad \Delta C = C_{\text{anti-OTA}^{\prime}\text{OTA}} - C_{\text{anti-OTA}} \quad (1)$$

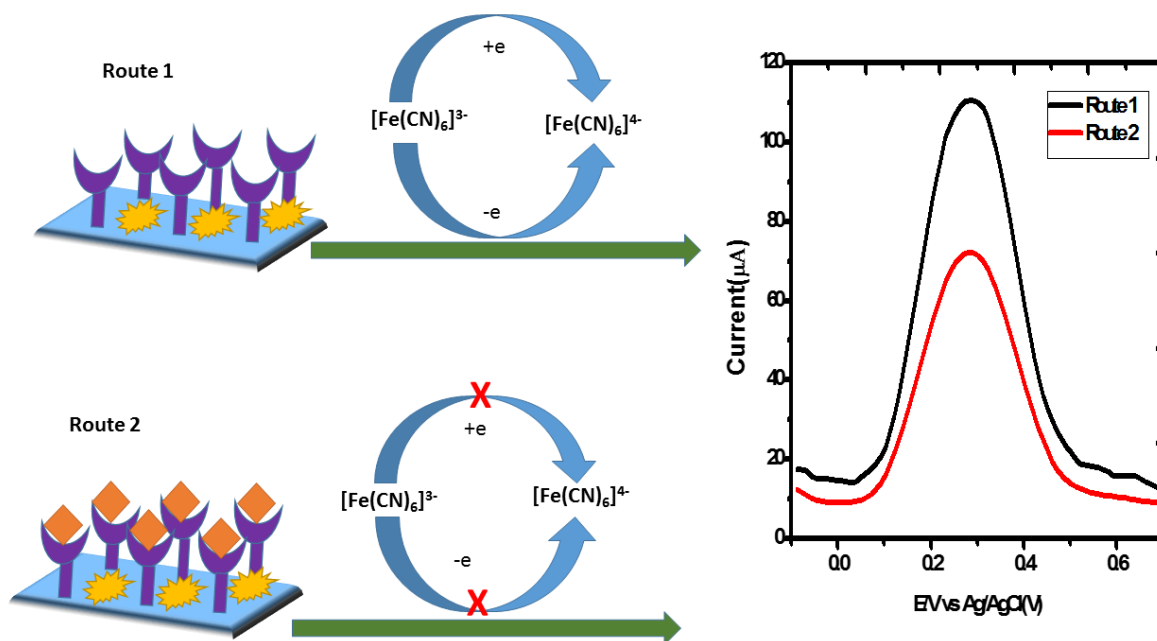
338 where $C_{\text{anti-OTA}^{\prime}\text{OTA}}$ is the value of the current after OTA coupling to the anti-OTA and $C_{\text{anti-OTA}}$
339 represents the value of the current of the native immunosensor. The immunosensor with
340 1.0 $\mu\text{g mL}^{-1}$ anti-OTA showed significant decrease in the current resulting in the LOD (0.25
341 ng mL^{-1}) and regression coefficient (R^2) of 0.9980 as shown in Fig. S3B and Table S1.
342 However, the immunosensor with 5.0 $\mu\text{g mL}^{-1}$ and 10 $\mu\text{g mL}^{-1}$ anti-OTA concentrations
343 showed a lower current with LODs and regression coefficients (R^2) of (0.39 ng mL^{-1} , 0.9850
344 and 0.44 ng mL^{-1} 0.9234 respectively. This is attributed to dense electrode surface with an
345 inadequate binding between the antigen and antibody to cause a current change. Therefore,
346 the thicker bioactive layer is the cause of a low performance of the immunosensor. Hence, 1.0
347 $\mu\text{g mL}^{-1}$ anti-OTA was chosen as the optimal concentration for the further characterization of
348 the immunosensor.

349

350

351 **3.2.7. Sensing mechanisms of the immunosensor**

352 In this work, the immunosensor was fabricated by covalently attaching antibody (Anti-
353 OTA) to the PdNPs coated CF electrode surface. The PdNPs are employed as the carriers of
354 the electrochemical capture probe to increase the change of peak currents. The ferricyanide
355 solution is used as a redox mediator to generate the electron flow between bulk solution and
356 working electrode as shown in Scheme 2. In the absence of OTA, the Anti-OTA offer a
357 significantly strong Faradaic current. However, in the presence of OTA the faradic current
358 decreases because, the formation of anti-OTA/OTA complex hinders the electron-transfer of
359 $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ [47, 48].



360
361 **Scheme 2:** The mechanism of the electrochemical immunosensor for the indirect detection of
362 OTA.

363

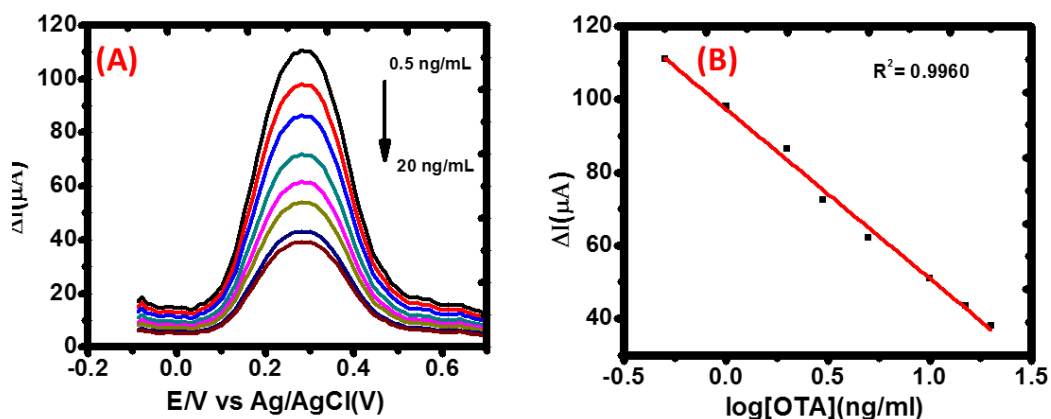
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366 3.2.8. Indirect quantification of OTA

367 The DPV response of BSA/anti-OTA/PdNPs/CF immunoelectrode recorded as a function of
368 OTA concentration ranging from 0.5 to 20 ng mL⁻¹ are depicted in Figure 3. The peak current
369 (ΔI) decreased with an increase in OTA concentration, showing the formation of
370 immunocomplex (antigen-antibody) at the electrode surface. This was established through the
371 interaction of antigen with the antibody (anti-OTA) absorbed onto the BSA/anti-
372 OTA/PdNPs/CF immunoelectrode which acts as an electron transporting layer [49, 50]. The
373 resulting DPV measurements were then used to plot the calibration curve for OTA. The
374 fabricated immunosensor BSA/anti-OTA/PdNPs/CF responds linearly to the logarithm
375 concentration of OTA ranging from 0.5 to 20 ng mL⁻¹ with LOD of 0.096 ng mL⁻¹ (3 \times se)/m
376 and a regression coefficient (R^2) of 0.9960 [Fig. 3B].

377 The biosensing parameters of the fabricated immunosensor were then compared to the
378 previously reported immunosensors for the detection of OTA (data given in Table S2). The
379 fabricated BSA/anti-OTA/PdNPs/CF immunoelectrode have the ability to detect a very low
380 concentration (96 pg mL⁻¹) of OTA as compared to other immunosensors [5, 8]. These results
381 show that PdNPs/CF materials provide high surface affinity to bind antibodies.



382

383 **Fig. 3.** (A) Electrochemical response studies of the BSA/anti-OTA/PdNPs/CF
 384 immunoelectrode as a function of OTA using DPV (Conditions: Scan rate: 30 mV s^{-1} ,
 385 Deposition time: 80 s, Pulse amplitude: 0.08 V and Pulse time: 0.03 s and (B) calibration
 386 curve between the magnitude of current and OTA concentration.

387

388 3.2.9. Recovery studies for the real samples

389 In order to investigate the ability of the fabricated BSA/anti-OTA/PdNPs/CF
 390 immunoelectrode, DPV responses were recorded in the presence of different concentrations of
 391 the spiked coffee samples. Table 1 shows the outcomes of the recovery studies for spiked
 392 sample in terms of electrochemical current. The DPV response was observed using five
 393 concentrations ($0.5, 1, 5, 10, 20 \text{ ng mL}^{-1}$), and the recovery was found in the range of 93.2-
 394 98.9 % with proportional error ranging from 1.0 to 6.8 %. These measured values of RSD and
 395 recovery were quite good and suggest that fabricated immunoelectrode is appropriate to be
 396 applied to OTA detection.

397

398 **Table 1:** Determination of OTA concentration in spiked samples using BSA/anti-OTA/PdNPs/CF
 399 immunoelectrode

Spiked concentration (ng/mL)	DPV response for		Recovery (%)	Proportional error (%)
	Spiked sample (μA)	OTA (μA)		
0.5	98.45	100.56	97.9	2.1
1	95.70	96.70	98.9	1.0
5	58.78	63.10	93.2	6.9
10	42.40	43.60	97.3	2.8
20	36.89	38.15	96.7	3.3

400

401 **3.2.10. Selectivity and shelf-life of immunoelectrode**

402 Selectivity and shelf-life are also very important parameters of immunoelectrodes. The
 403 selectivity of the immunoelectrode was investigated by monitoring the DPV response of
 404 BSA/anti-OTA/PdNPs/CF in the presence of the interferences. Different interferences such as
 405 BSA, Aflatoxin B1 and L-Tryptophan (10 ng mL^{-1}) were mixed with OTA (1 ng mL^{-1}), and the
 406 DVP response was assessed (Fig. S4B). There was no significant change in the DPV response
 407 after the interaction of the immunoelectrode with interfering compounds. This indicates that
 408 the fabricated immunoelectrode is only selective to OTA detection. The DPV technique was
 409 used to investigate the shelf-life of immunoelectrode on a regular interval of seven days up to
 410 three weeks using the optimized parameters. The fabricated immunoelectrode shown in Fig.
 411 S4A shows that is stable up to at least three weeks, with a slight change in current value (99.6
 412 %) was observed. This suggests that BSA/anti-OTA/PdNPs/CF immunoelectrode is highly
 413 stable.

414

415 **3.3. Reproducibility and repeatability of immunoelectrode**

416 The reproducibility of the BSA/anti-OTA/PdNPs/CF immunoelectrode was studied using
417 the interassay methods where DPV response was studied for six individual immunoelectrodes
418 prepared independently as shown in Fig.S5A. The value of relative standard deviation (RSD)
419 was found to be 5.6 %. Additionally, the repeatability of the immunoelectrode was
420 investigated using one immunoelectrode for six successive measurements, and the results
421 showed a good standard deviation of 1.4% as shown in Fig. S5B. The repeatability results
422 show that the fabricated immunosensor can be reusable. In fact, the repeatability of the
423 fabricated immunosensor shows that the standard deviation is below 2%, after six successive
424 measurements conducted on one immunosensor. Further study would however be needed for
425 a more precise understanding the reusability of the fabricated immunosensor.

426

427 **Conclusion**

428 In this study, we reported the fabrication and the characterization of a novel and highly
429 efficient electrochemical immunosensor for the selective detection of OTA. Atomic layer
430 deposition has been successfully used as an efficient route to produce highly dispersed PdNPs
431 onto the surface of carbon felt (CF) electrodes, and the BSA and the anti-OTA antibodies
432 were then grafted onto the composite structure via a carbodiimide cross linkage route.
433 Subsequently, the developed immunosensor was used to detect the OTA in coffee samples.
434 The fabricated BSA/anti-OTA/PdNPs/CF immunosensor showed outstanding electrochemical
435 performances such as a wide detection range of 0.5-20 ng mL⁻¹ and a LOD of 0.096 ng mL⁻¹
436 towards the detection of OTA. This study also revealed that the PdNPs accelerate the electron

437 transfer rate on the large surface area electrodes. Additionally, the immobilization of anti-
438 OTA on the surface of the electrodes offer specific intrinsic immuno-recognition, with an
439 improved binding efficiency, wettability property and enhanced selectivity of the sensor.
440 Finally, this study also revealed that the fabricated immunosensor were selective to OTA in
441 the presence of interfering compounds and that the sensors were stable for up to three weeks.
442 The results presented in this work open prospects for new sensing routes for molecules of
443 interest in food products.

444

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