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Smart Electrochemical Immunosensing of Aflatoxin B₁ Based on a Palladium Nanoparticle-Boron Nitride-Coated Carbon Felt Electrode for the Wine Industry

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Abstract

This study describes the fabrication of an aflatoxin (AFB₁) electrochemical immunosensor by immobilization of an anti-AFB₁ antibody-bovine serum albumin (BSA) conjugate on a layer of L-cysteine (L-Cys) used to link the antibody to the palladium nanoparticle-boron nitride (PdNP-BN)-modified carbon felt (CF) electrode. Each step of the immunosensor fabrication was characterized using cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). These two analyses demonstrated that BN acted as a substrate for PdNPs and also as an electroactive material that enhanced the immunosensor electrical conductivity. Different features (antibody concentration, pH, incubation time) were optimized to enhance the immunosensor analytical performance. In these conditions, the fabricated immunosensor displayed a large linear range (1-10 ng mL⁻¹; R² = 0.9987), low limit of detection (0.834 ng mL⁻¹ AFB₁), and was stable for up to 3 weeks. The fabricated electrochemical immunosensor could detect AFB₁ in wine samples with recovery levels of 93-106%. Lastly, the Monte Carlo simulation studies confirmed the stronger binding of the anti-AFB₁ antibody-BSA conjugate to the L-Cys/PdNP-BN/CF electrode surface, and molecular docking confirmed the docking of BSA subdomain II to the anti-AFB₁ antibody.

Keywords: Aflatoxin B₁; wine sample; Atomic Layer Deposition, palladium nanoparticles; boron nitride; immunosensor

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1. Introduction

Wine is one of the most important alcoholic beverages in many societies. Besides its very well-known adverse effects, wine may also contain various toxins and contaminants because grapes can be contaminated by very harmful mycotoxins, such as aflatoxins [1]. Therefore, their presence in wine must be monitored to ensure the product safety. Aflatoxins are highly toxic substances produced by some molds, such as *Aspergillus flavus* and *Aspergillus parasiticus* [2-4]. There are different aflatoxin types (AFB₁, AFB₂, AFG₁ and AFG₂) as well as two aflatoxin metabolites (AFM₁ and AFM₂) [5]. AFB₁ is the most toxic, mutagenic, teratogenic, and carcinogenic aflatoxin [6, 7]. According to the International Agency for Research on Cancer (IARC), AFB₁ is a group 1 carcinogen [8, 9]. AFB₁ has been detected in many different food products, such as wine, grape juice, rice, pasteurized milk, and maize [2, 3, 6, 10-12]. The maximum permissible AFB₁ level in food samples differs among countries. For instance, this level is 5 µg/kg in South Korea and 2 µg/kg in the European Union [13]. Therefore, AFB₁ must be quantified to avoid the supply and ingestion of contaminated food products.

AFB₁ can be detected using analytical methods (e.g. liquid chromatography-mass spectrometry [14], enzyme-linked immunosorbent assay [15], thin-layer chromatography [16], high-performance liquid chromatography [17]) and optical methods [18, 19]. These techniques are sensitive and specific, but they are laborious and require well-trained operators, extensive sample pre-treatment, and expensive instruments. Thus, rapid, sensitive, selective and cheaper techniques to precisely quantify AFB₁ levels in foods and beverages are urgently needed to be used by industries and inspection agencies. In the last few decades, a significant number of electrochemical sensing methods have been proposed for AFB₁ detection: electrochemical sensors [20], electrochemiluminescence [21], photoelectrochemical sensors [22], and electrochemical immunosensors [23]. Electrochemical immunosensors display very good sensitivity, efficiency, specificity, and consistency. They are also cheap and easy to use. The

goal of the present study was to design and fabricate smart 2D materials to improve sensing platforms for AFB₁ detection in wine samples.

When designing electrochemical immunosensors, electrode modification is a well-known procedure for modifying the electrical conductivity and specific surface area to increase the immunosensor sensitivity. Recently, many studies have focused on palladium nanoparticles (PdNPs) due to their conductive features, large surface area, catalytic, and electronic properties [24, 25]. Furthermore, PdNPs have become a common material in the biosensing field [26, 27]. Previous studies have shown that the nanoparticle (NP) size should be reduced to improve the catalytic activity. The NP supporting material also plays a crucial role [28, 29]. Carbon and high melting point oxides are frequently used as supporting material, but boron nitride (BN)-based materials are a new interesting alternative [30-32]. Indeed, BN is a promising supporting substrate for electrochemical sensors due to its remarkable features, such as high surface area, low toxicity, and good catalytic properties [33, 34]. Moreover, the growth of metal NPs on the BN support can improve the sensing of different analytes [35]. Therefore, using BN as substrate for PdNP deposition appears to be a promising route for electrochemical sensor development. There are several methods for growing NPs on the BN support: electrodeposition [36], hydrothermal deposition [37], chemical vapour deposition [38], spray drying and pyrolysis [39]. They require high temperatures, toxic precursors, and complex and long experimental methods. For this reason, atomic layer deposition (ALD) is now considered the most effective method for BN and PdNP synthesis with excellent uniformity [29]. It also provides NPs and films with high conformality aspect ratio and controlled thickness [40]. Therefore, for many applications, such as biosensors, thin films and membranes, ALD is an effective method for depositing films and NPs [40-42].

Besides the substrate material, the method used to fabricate the electrochemical immunosensor also strongly contributes to enhance its analytical performance.

L-cysteine (L-Cys) is an amino acid that contains an amino, sulfhydryl and carboxylic functional group. L-Cys has distinctive properties and is extensively used in biological, environmental and electrochemical research [43-45]. Furthermore, L-Cys has been used for providing a self-assembly layer and for changing the surface of noble metal NPs [46, 47]. L-Cys harbours different functional groups, such as the -SH groups that binds to the NP surface, and the (COOH) and (-NH₂) groups that stabilize NPs [48] and offer a hydrophilic interface for the analyte-NP surface interaction [49, 50].

In a previous study, we described the synthesis of PdNPs on carbon felt (CF) using ALD for bio-sensing applications [51]. Specifically, we developed an electrochemical immunosensor for AFB₁ detection by growing PdNPs on a thin layer of BN, using L-Cys for palladium-sulphur bond (Pd-S bonds) formation for the anti-AFB₁ antibody attachment.

The current study focused on the fabrication of an electrochemical immunosensor for AFB₁ detection in wine samples using L-Cys/PdNP-BN followed, for the first time, by computational modelling of the anti-AFB₁ antibody binding to the L-Cys/PdNP-BN/CF electrode surface. Our study revealed that the fabricated immunosensor is a proficient tool for the detection of AFB₁ in wine samples.

2. Experimental

2.1. Materials and reagents

CF MGL 190, fuel cell earth (10 Draper St. Unit 32 Woburn, MA, MA 01801 www.Fuelearth.com), ammonia (NH₃) (Air Liquide Paris, France), L-Cys (99.0% purity), *N*-hydroxysuccinimide (NHS; 98.0% purity), *N*-ethyl-*N*-(3-dimethylaminopropyl carbodiimide (EDC; 97.0% purity), AFB₁ (3.79 μg g⁻¹ in acetonitrile), ochratoxin A (OTA; 1 mg mL⁻¹ in DMSO, 98.0% purity), ethanol (CH₃CH₂OH; 95.0% purity), potassium ferricyanide ([Fe(CN)₆]³⁻, 99.0% purity), potassium ferrocyanide ([Fe(CN)₆]⁴⁻, 98.5-102.0% purity),

acetonitrile (CH_3CN ; 99.8% purity), boron tribromide (BBr_3 ; 99.9% purity), dipotassium hydrogen phosphate (K_2HPO_4 ; 98.0% purity), potassium dihydrogen phosphate (KH_2PO_4 ; 99.0% purity), sulphuric acid (H_2SO_4 ; 95.0-98.0% purity), formaldehyde solution (CH_2O ; ACS reagent, 37 wt. % in H_2O , with 10-15% of methanol as stabilizer), bovine serum albumin (BSA; 98.0% purity), palladium (II) hexafluoroacetylacetonate [$\text{Pd}(\text{C}_5\text{HF}_6\text{O}_2)_2$, 99.0% purity], and rabbit anti-AFB₁ polyclonal antibodies were purchased from Sigma Aldrich France. Ultrapure water (Mill-Q, Millipore, 18.2 M Ω resistivity) was used for all experiments.

2.2.Apparatus and characterization

The measurements using linear sweep voltammetry (LSV) and cyclic voltammetry (CV) were performed on the Solartron 1286 electrochemical interface (TICS International Ltd., UK), using a three-electrode system: working electrode (modified CF: 3.5 cm x 0.7 cm x 0.3 cm in size), auxiliary electrode (graphite rod), and reference electrode (Ag/AgCl). An iS50 ATR Thermo Scientific spectrophotometer (range: 400-4000 cm^{-1}) was used to acquire attenuated total reflectance (ATR) spectra. The SP-150 EC-LAB Electrochemistry chemical workstation (VSP Potentiostat from BioLogic Science Instruments, France) was used for electrochemical impedance spectroscopy (EIS) analyses carried out at ~ 25.0 °C. The fabricated immunosensor was morphologically characterized using an atomic force microscope (AFM, Veeco Nanoscope Dimension 3100). A VG ESCALAB 250 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for X-ray photoelectron spectroscopy (XPS), and a JEOL ARM 200F high-resolution transmission electron microscope (200 kV) with an EDX analyser (JED2300, at least 30 accumulations, matrix 512×512 points in STEM mode) for the high-resolution transmission electron microscopy (HR-TEM) analysis.

2.3. Antibody and sample preparation

The 0.1M phosphate buffer saline (PBS) solution (pH 7.5) was prepared by mixing an equimolar ratio of dipotassium hydrogen phosphate and potassium dihydrogen phosphate. This PBS solution was used to dilute the anti-AFB₁ antibody (1 $\mu\text{g mL}^{-1}$). AFB₁ was dissolved in PBS with 10% methanol and stored at 20 °C. A BSA solution (1 $\mu\text{g mL}^{-1}$ in PBS) was used as blocking agent.

The red wine samples were prepared following the procedure described by Goud and co-workers [9]. Wine was mixed with PBS and acetonitrile (1:3:1). The mixture was then sonicated for 1h, centrifuged at 5000 rpm for 1h, and filtered using a micro-filter with pore size 0.1-10 μm . The filtrate was spiked with different concentrations of AFB₁ and used for recovery studies with the fabricated immunosensor.

2.4. CF pre-treatment

CF were pre-treated to change their hydrophobic nature to hydrophilic using the method described by Kosimaningrum and co-workers [52]. To eliminate the impurities that might be present on the surface, CF were ultra-sonicated in ethanol for 2h, washed with deionized distilled water, and oven-dried at 70 °C overnight. Then, CF were electrochemically pre-treated in 1M H₂SO₄ by CV from 0 to 1.5 V versus Ag/AgCl for 30 cycles at 20 mV s⁻¹. Finally, samples were thoroughly washed with deionized distilled water until neutral pH and oven-dried at 70 °C.

2.5. Synthesis of PdNPs on BN by ALD

A homemade low-pressure ALD reactor was used to deposit BN and PdNPs onto the CF surface. BN deposition was carried out at 750 °C, using BBr₃ and NH₃ as precursor and co-reactant, respectively [53]. The process consisted in: BBr₃ pulse for 0.1s, exposure for 5s, purge

with inert gas for 15s, NH₃ pulse for 3s exposure to gas for 5s, and purge for 15s. The obtained samples are called BN/CF hereafter.

For PdNP deposition on BN/CF (PdNP-BN/CF hereafter), the hot-wall chamber temperature was maintained at 220 °C, while Pd(C₅HF₆O₂)₂ was heated to 70 °C in a sealed stainless steel bottle to reach a sufficiently high vapor pressure. The inlet lines were heated to 100 °C to limit condensation. PdNPs (200 cycles) were deposited on BN/CF following the procedure recently described by our team [51]. One Pd cycle was completed by first dosing Pd(C₅HF₆O₂)₂, followed by exposure for 15s and purge with argon gas for 10s. Then, formalin was pulsed for 1s, followed by exposure for 15s, and a final purge for 60s.

2.6. Fabrication of the label-free BSA/anti-AFB₁/L-Cys/PdNP-BN/CF immunosensor

Fig. 1 summarizes the fabrication process of the electrochemical immunosensor for AFB₁ detection in wine samples. First, the PdNP-BN/CF electrode was immersed in a 10 mmol L⁻¹ L-Cys solution at room temperature overnight to form Cys/NP thiolate bonds. After washing with PBS to remove unbound L-Cys molecules, 20 μL of 0.4 mol L⁻¹ EDC and 0.1 mol L⁻¹ NHS (1:3 ratio) were dropped on the L-Cys/PdNP-BN/CF electrode at room temperature for 50 min to convert the terminal carboxylic groups to active NHS esters [54]. Then, the modified electrode was rinsed with PBS, and 20 μL of anti-AFB₁ antibody solution (1.0 μg mL⁻¹ in PBS) was spread over the L-Cys/PdNP-BN/CF electrode surface and incubated at 4.0 °C for 2h. The resulting anti-AFB₁/L-Cys/PdNP-BN/CF electrode was rinsed with PBS to remove the excess antibody. Then, to block the non-active sites, 10 μL of BSA (10%, m/v) was dropped on the anti-AFB₁/L-Cys/PdNP-BN/CF electrode and left at room temperature for 20 min (BSA/anti-AFB₁/L-Cys/PdNP-BN/CF). When not used, electrodes were stored at 4 °C.

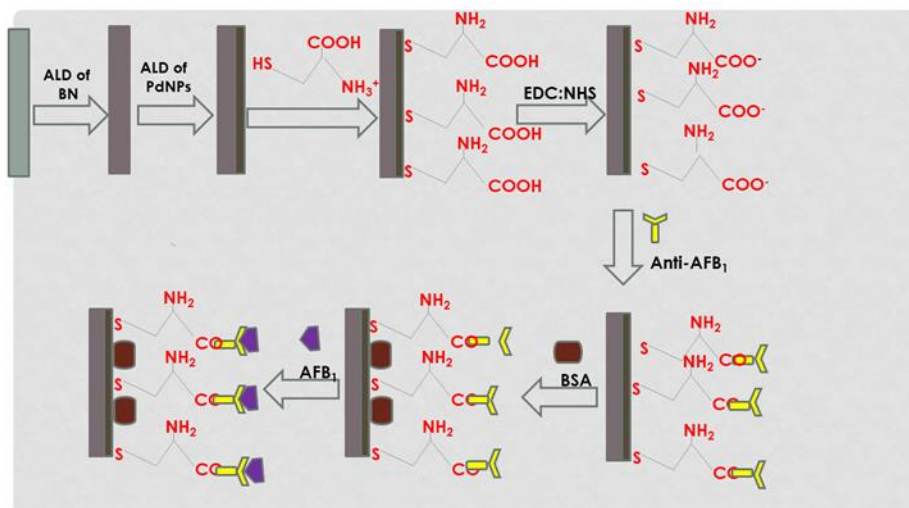


Fig. 1: Schematic representation of the fabrication procedure of an electrochemical immunosensor for AFB₁ detection in wine samples.

2.7. Biorecognition experiments

The biorecognition experiments were carried out by dipping the BSA/anti-AFB₁/L-Cys/PdNP-BN/CF electrode in a cell that contained 20 mL PBS (pH 7.5). Then, 10 μ L of AFB₁ solution at different concentrations (1.0, 2.0, 4.0, 6.0, 8.0 and 10 ng mL⁻¹) was added to the cell for 30 min. Similar experiments were performed using wine samples that contained different AFB₁ concentrations. All experiments were done three times.

2.8. Safety considerations

As AFB₁ is a potent carcinogen, protective gloves, safety goggles, and disposable face mask were used during the experiments. The contaminated glassware was soaked in a sodium hypochlorite solution (5.25%) for 72h before washing.

3. Computational modelling

3.1. Molecular docking of the anti-AFB₁ antibody with BSA (BSA-anti-AFB₁)

The 3D structure of the monoclonal antibody against AFB₁ was obtained from the RCSB Protein Data Bank (1IGY). This entry contains four unique chains (A-D) and 12750 atoms, including 2498 hydrogen atoms and 0 deuterium atoms [55]. The crystal structure of BSA (RCSB Protein Data Bank; 3v03) contained two unique chains (A and B) and 8828 atoms (0 hydrogen and 0 deuterium) [56]. The antibody was docked to BSA using the ClusPro protein-protein docking server, with advanced options set to antibody mode [57], and the results were visualized using Discovery Studio Visualizer.

3.2. Nanocluster construction

The CF, BN and Pd nanoclusters were built using the Build option and the standard library of parameters of the BIOVIA Materials Studio software package [58]. The 3D nanoclusters were energy-minimized and their geometry optimized with the Forcite module and the UNIVERSAL force field.

3.3. Monte Carlo simulations of the BSA/anti-AFB₁/L-Cys/PdNP-BN/CF electrode adsorption capacity

Monte Carlo simulations were used to determine the lowest energy configurations of the L-Cys/PdNPs-BN/CF electrode (the adsorbate) on the BSA-anti-AFB₁ surface (the substrate) at progressively decreasing temperatures. The Adsorption Locator module in the Material Studio package (as preparatory and screening tool) and the force-field method were used to rank the obtained energy configurations, and to identify the preferred adsorption sites. Monte Carlo simulations of the adsorbate-substrate configurational space at progressively decreasing temperatures, following a simulated annealing protocol, were performed to identify adsorption configurations. The identified adsorbate-substrate configurations were ranked using our

previously described protocol [59, 60], and the lowest adsorption energy conformers were optimized with the Forcite module to obtain a stable conformation.

4. Results and discussion

4.1. Structural characterization of PdNP-BN

BN/CF and PdNP-BN/CF were successfully prepared by ALD. HR-TEM analysis of PdNP-BN/CF samples (**Fig. 2A**) indicated that PdNPs were well dispersed on the CF surface with a mean diameter of 6 nm (**Fig. 2B**). It also confirmed the deposition of a conformal and continuous ultrathin BN layer of ~2 nm on the CF surface (Fig. S1B-C). The number of ALD cycles to obtain the best BN thickness was studied, and was found to be 25 cycles. The Fast Fourier transform (FFT) spectra (**Fig. 2C**) confirmed the crystallinity of the deposited PdNPs. Energy-dispersive X-ray spectroscopy (EDS) maps of elements (**Fig. 2D-F**) confirmed the existence of B, N and Pd in the studied samples. The chemical state of C, B, N, O and Pd in modified CFs, evaluated by XPS, showed the presence of C, B, N, O and Pd in PdNP-BN/CF (**Fig. 2G**). The fitted B1s spectra displayed a peak centred at 192.5 eV (**Fig. 2H**) that was assigned to B–N bonding. In the N 1s spectra (**Fig. 2I**), the initial peak was divided in two major peaks that were assigned to N–O (398.2 eV) and B–N (400.1 eV). The Pd 3d_{3/2} and 3d_{5/2} binding energies were 340.9 and 335.6 eV, respectively (**Fig. 2J**). The deconvoluted peaks were assigned to Pd⁰ and PdO_x, and the metal Pd⁰ was a major phase. These results are similar to those previously published by Weber et al [61]. The presence of PdO_x species is related to the fact that BN attracts electrons from PdNPs. The O 1s and C 1s spectra are shown in Fig. S2.

Doping level is crucial because carrier density is crucial for fine-tuning the performance of the material. It has been established that doping Pd NPs with either electron-donating (nitrogen) or electron-withdrawing (boron) groups significantly alters the electrical properties of the NPs.

When the Pd NP is doped with electron-withdrawing (electron acceptors, p-type) or electron-donating (n-type, electron donors) groups, the electron density varies, which also influences the NP's electrochemical properties.

4.2. Characterization of the fabricated immunosensor (BSA/anti-AFB₁/L-Cys/PdNP-BN/CF)

AFM was used for the topographical and morphological analysis of the modified electrode because it is one of the most efficient techniques to evaluate the topographical and surface roughness. The 2D topographical AFM images of the L-Cys/PdNP-BN/CF electrode (Fig. S3A), obtained in static mode, showed the electrode surface topography after L-Cys modification. The surface layer was uniform and widespread without aggregates or layer faults, with a surface roughness (R_z) of 0.6028 μm . Upon immobilization of the anti-AFB₁ antibody on the L-Cys/PdNPs-BN/CF surface (Fig. S3B), the surface appeared more globular, indicating its protein nature [12]. The surface roughness increased to 1.2552 μm . This demonstrates that the homogeneity of the L-Cys layer allowed the uniform antibody binding to the functional moiety [62]. AFM revealed that this is an efficient approach to immobilize antibodies for immunosensor production. ATR spectroscopy was used to identify the functional groups at the immunosensor surface (**Fig. 2K**). The ATR spectra of PdNP-BN/CF (Fig. S4A) showed two bends at 825 cm^{-1} and 1572 cm^{-1} that are the B-N in plane stretching mode and the out of plane vibration of h-BN, respectively [63]. When the electrode was modified with L-Cys (**Fig. 2K**; curve i; L-Cys/PdNP-BN/CF), the spectrum showed four peaks. The peaks at 1446 cm^{-1} and 1365 cm^{-1} were assigned to the asymmetric and symmetric stretching of COO⁻, and those at 1528 cm^{-1} and 1299 cm^{-1} to the bending mode of -NH₂ and to the C-N bend, in agreement with the findings by Panhwar and co-workers [64]. The characteristic peak confirmed that L-Cys was modified on the electrode surface. A very weak thiol group (-SH) peak at 2585 cm^{-1} indicated that L-Cys was present on the electrode surface [65]. Antibody docking on L-

Cys/PdNP-BN (curve ii; anti-AFB₁/L-Cys/PdNP-BN/CF) resulted in peaks at 3001 cm⁻¹, 1689 cm⁻¹, and 866 cm⁻¹ due to amide bond (N-H) deformation, stretching, and -NH₂, respectively, on the electrode surface [51, 66]. These data confirmed the antibody immobilization. After incubation with BSA (curve iii; BSA/anti-AFB₁/L-Cys/PdNP-BN/CF), the -NH₂ group disappearance from the spectrum confirmed that the non-specific sites on the anti-AFB₁/L-Cys/PdNPs-BN/CF immunosensor were blocked by BSA [67].

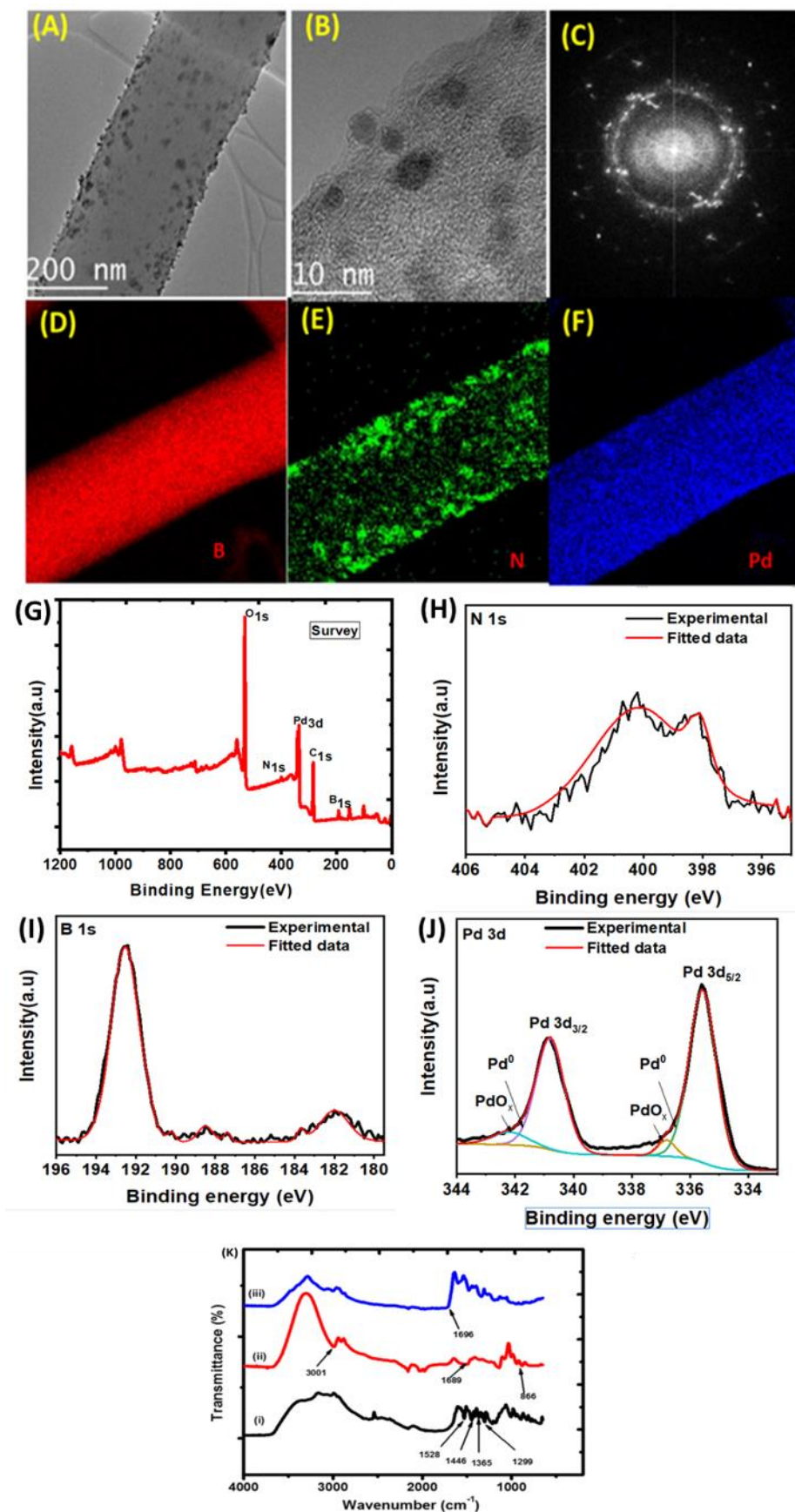


Fig. 2: (A-B) TEM micrographs of PdNP-BN at low and high magnification; (C) Fast Fourier transform spectrum of PdNP-BN; (D-F) EDS maps of B, N and Pd, respectively; same sample as in (A); (G) XPS survey spectrum of PdNP-BN/CF; (H-J) High-magnification XPS spectra

of B 1s, N 1s and Pd 3d, respectively; (K) ATR spectra of (i) L-Cys/PdNP-BN/CF, (ii) anti-AFB₁/L-Cys/PdNP-BN/CF, and (iii) BSA/anti-AFB₁/L-Cys/PdNP-BN/CF.

4.3. Electrochemical characterization of the electrode interfaces

CV and EIS in a 0.1M KCl and 5.0 mM [Fe(CN)₆]^{3-/4-} (the redox probe) solution were used to characterize each step of the immunosensor fabrication process (**Fig. 3A** and **B**). For the PdNP-BN/CF sample (curve i), the electrochemical reaction was quasi-reversible with a peak separation at ~110 mV and 20 mV/s, and an anodic current (I_{pa}) of 1.45 μ A. The current increase due to BN catalytic behaviour facilitated the electron transfer. L-Cys adsorption on the electrode surface led to a I_{pa} decrease to 0.73 μ A (curve ii). L-Cys reduced the resistance of the electrostatic repulsion between the -COOH of L-Cys and [Fe(CN)₆]^{3-/4-} negative charge [68]. Upon immobilization of the anti-AFB₁ antibodies to produce the anti-AFB₁/L-Cys/PdNP-BN/CF electrode, I_{pa} increased to 0.97 μ A (curve iii). This indicates that the anti-AFB₁ antibodies act as a mediator activity between L-Cys/PdNP-BN and CF. The shorter electron tunnelling distance between antibodies and electrode explains the peak current increase [69]. Non-specific site blocking with BSA to form the BSA/anti-AFB₁/L-Cys/PdNP-BN/CF sensor resulted in a I_{pa} decrease to 0.50 μ A (curve iv). This was caused by BSA adsorption on the surface. EIS is among the best tools to analyse the modified electrode surface [68]. The EIS shape includes high frequencies and low frequencies. The high frequencies contain a semicircle where electron transfers occur, whereas the low frequencies contain a linear portion where the electrochemical process occurs. The modified Randles circuit (inset in **Fig. 3B**) was used for fitting the impedance data. The circuit included the ohmic resistance of the electrolyte solution (R_s), double layer capacitance (C_{dl}), charge transfer resistance (R_{ct}), and Warburg impedance element (Z_w). Chen and co-workers showed that R_s and Z_w are not affected by reactions taking place on the electrode surface. Conversely, C_{dl} and R_{ct} are influenced by the insulating features and the electrode interface, respectively [70]. **Fig. 3B** shows the Nyquist plots for the

electrode at different modification steps. R_{ct} and Z_w were parallel with C_{dl} . The bare CF showed a small semicircle diameter with $R_{ct} = 9 \Omega$, implying a low charge transfer resistance (Fig. 4SB). In PdNPs-BN/CF (Fig. 3B; curve i), R_{ct} slightly increased to 84Ω , due to the excellent electrocatalytic activity of PdNPs and BN. When L-Cys was assembled onto the PdNP-BN/CF surface, R_{ct} increased to 1200Ω (curve ii). This shows that L-Cys formed a large barrier to the interfacial charge transfer, as indicated by the increasing semicircle diameter in the spectrum. Similar results were reported by Fan and co-workers [71]. When the anti-AFB1 antibody was covalently bound to L-Cys, R_{ct} decreased to 567Ω (curve iii), confirming the antibody immobilization on the surface of the modified electrode. R_{ct} decreased also after BSA immobilization onto the modified electrode (curve iv), demonstrating the successful fabrication of the immunosensor.

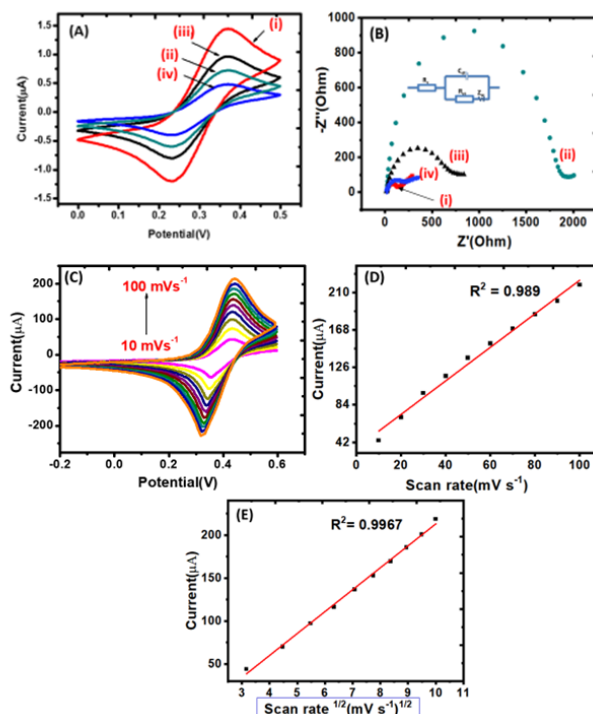


Fig. 3: (A) Cyclic voltammograms and (B) Nyquist plots for (i) PdNP-BN/CF, (ii) L-Cys/PdNP-BN/CF, (iii) anti-AFB₁/L-Cys/PdNP-BN/CF, and (iv) BSA/anti-AFB₁/L-Cys/PdNP-BN/CF. Conditions: solution of 0.1M PBS, 0.1M KCl and 5 mM [Fe(CN)₆]^{3-/4-}, pH 7.5, scan rate of 20 mV s⁻¹. (C) Relationship between peak current and scan rate. (D-E) Graphs

showing the relationship between cathodic peak current and scan rate (D) or the scan rate square root (E) for the BSA/anti-AFB₁/L-Cys/PdNP-BN/CF sensor in the same solution as for (A-B).

4.4. Investigation of the scan rate effects on BSA/anti-AFB₁/L-Cys/PdNP-BN/CF

To determine the electrochemical reaction mechanism of the developed immunosensor, the effect of different scan rates (v) (from 10 to 100 mV s^{-1}) with BSA/anti-AFB₁/L-Cys/PdNP-BN/CF was studied using CV (**Fig. 3C**). The linear increase of the current with the scan rate, in the redox probe, according to the equation $I_{\text{pa}} (\mu\text{A}) = 1.882 v (\text{mV s}^{-1}) + 35.689$ ($R^2 = 0.989$), suggested a diffusion-controlled reaction (**Fig. 3D**). This was confirmed by the linear relationship between the scan rate square root and the cathodic current (**Fig. 3E**), in agreement with the observed diffusion-controlled reaction [72, 73].

4.5. Optimizing the experimental conditions

To improve the immunosensor analytical performance, key parameters (i.e. pH of the PBS solution, L-Cys concentration, incubation time for the specific binding to the target material, antibody concentration and immobilization time) were optimized using 6.0 ng mL^{-1} AFB₁. The pH effect was optimized by testing PBS solutions at different pH (from 5.5 to 8.5). The immunosensor peak current increased in the presence of PBS solutions up to $\text{pH} = 7.5$ and then decreased (**Fig. 4A**). This shows that a highly basic environment denatures the activity of the immobilized antibodies. Additionally, the Sigma-Aldrich safety data sheet indicates that the pH of the anti-AFB₁ antibody is 7.4. This means that the antibody maintains its activity and stable combination with the antigen at neutral pH. Therefore, the PBS solution at $\text{pH} 7.5$ was chosen for AFB₁ detection. Then, various L-Cys concentrations (5.0, 10, 15, 20, and 25 mmol L^{-1}) were tested. The peak current increased when L-Cys concentrations from 5.0 to 10 mmol L^{-1} were used, and then decreased, due to electrode saturation (**Fig. 4B**). The incubation time role was tested by varying the incubation time of the immunosensor (**Fig. 4C**). As the peak current progressively increased and reached a plateau at 30 min, this time duration was selected

as optimal. The concentration of antibodies immobilized on the electrode surface influences the sensor sensitivity due to the availability of more or fewer binding sites for antigens. Four different anti-AFB₁ antibody concentrations were tested (0.5, 1.0, 5.0 and 10 $\mu\text{g mL}^{-1}$) (Fig. 4D). The peak current increased when 0.5 to 1.0 $\mu\text{g mL}^{-1}$ antibodies were immobilized, and then progressively decreased with increasing concentrations. Therefore, the anti-AFB₁ antibody concentration of 1.0 $\mu\text{g mL}^{-1}$ was used for all experiments.

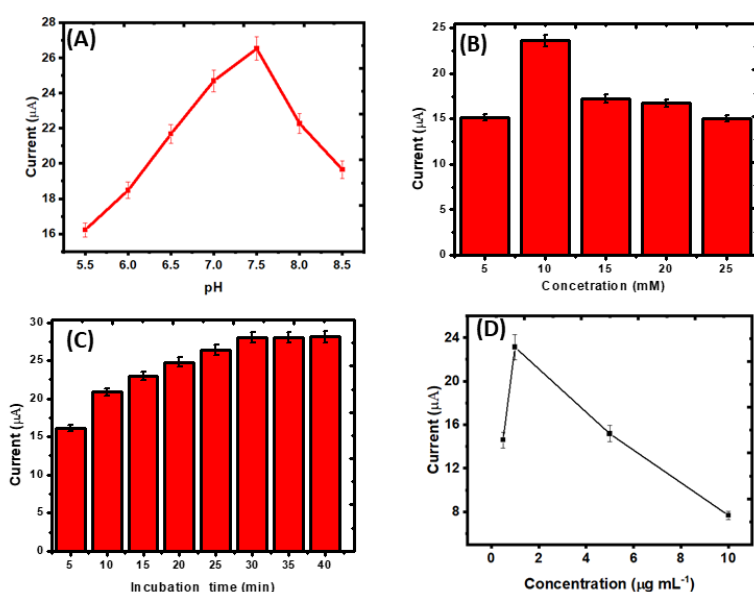


Fig. 4: Effects on the peak current of BSA/anti-AFB₁/L-Cys/PdNP-BN/CF in the presence of 6 ng mL^{-1} AFB₁ of the following parameters (A) pH, (B) L-Cys concentration, (C) incubation time, and (D) anti-AFB₁ antibody concentration. The electrochemical experiments were performed in a solution of 0.1M KCl, 0.1M PBS (pH 7.4) and 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$.

4.6. Sensing mechanisms of the immunosensor

In this work, the immunosensor was fabricated by immobilizing L-Cys on the PdNP-BN/CF electrode surface to form thiolated bonds. The PdNP-BN surface was used as a carrier of the electrochemical capture probe to increase the peak current change. Then, the terminal carboxylic groups present in the thiolated bond were converted to NHS esters by attaching the crosslinker (EDC:NHS) to L-Cys/PdNP-BN/CF. The anti-AFB₁ antibody was then covalently attached to the NHS esters, followed by incubation with BSA to block the immunosensor non-

specific sites. The ferricyanide solution was used as redox mediator to generate the electron flow between the bulk solution and the working electrode. In the absence of AFB₁, the anti-AFB₁ antibodies gave a significantly strong faradaic current. Conversely, in the presence of AFB₁, the faradaic current decreased because the formation of anti-AFB₁ antibody-AFB₁ complexes hinders the electron transfer of Fe(CN)₆³⁻/Fe(CN)₆⁴⁻ [74, 75].

4.7. Analytical characteristics of the BSA/anti-AFB₁/L-Cys/PdNP-BN/CF immunosensor

The performance of the fabricated immunosensor was verified using AFB₁ at different concentrations (1.0 to 10 ng mL⁻¹) and the optimized parameters. The LSV curves (from triplicate measurements) showed that the peak current decreased with higher AFB₁ concentrations (**Fig. 5A**). This is due to hindrance of electron transfer to the electrode after AFB₁ adsorption onto the BSA/anti-AFB₁/L-Cys/PdNP-BN/CF immunosensor. Specifically, at the tested concentrations, the peak current displayed a linear relationship with AFB₁ detection ($R^2 = 0.9987$), and the limit of detection (LOD) was 0.834 ng mL⁻¹ ($3 \times se$)/m (**Fig. 5B**). These results are in agreement with the reference range and indicate that the immunosensor can be used to detect AFB₁ in alcoholic beverages. The immunosensor precision was determined by consecutive determination of 6.0 ng mL⁻¹ of AFB₁ for 20 times using the same experimental set-up and immunosensor. This experiment gave a relative standard deviation (RSD) of 1.5%. Reproducibility was assessed using six different electrodes fabricated independently and gave an RSD of 1.2%. These results demonstrate that the fabricated immunosensor possesses good reproducibility and tolerable precision (Fig. S6A). Moreover, after storage in an airtight container at 4 °C for 3 weeks, the immunosensor peak current was 81.74% of the initial value, indicating good stability (Fig. S6B). The specificity of label-free immunosensors generally is limited, possibly due to non-specific adsorption of foreign compounds on the sensing surface. Therefore, the detection by the immunosensor of OTA and AFB₁ (1 ng mL⁻¹ and 10 ng mL⁻¹) individually and mixed was evaluated. A negligible peak

current was observed when the immunosensor was incubated with OTA or the mixture, even at the higher concentration. Conversely, the peak current noticeably changed in the presence of AFB₁. These results show that the fabricated immunosensor is specific for AFB₁ (Fig. S6C). Compared with other electrochemical immunosensors for AFB₁ detection (Table S1), the fabricated immunosensor had a low detection limit and acceptable detection range. Its good electrochemical performance could be explained by the wide surface area, good biocompatibility, and good signal electrochemical amplification of the L-Cys/PdNP-BN composite. In addition, the large anti-AFB₁ antibody amount loaded onto the L-Cys/PdNP-BN surface enhances the possibility of antigen binding.

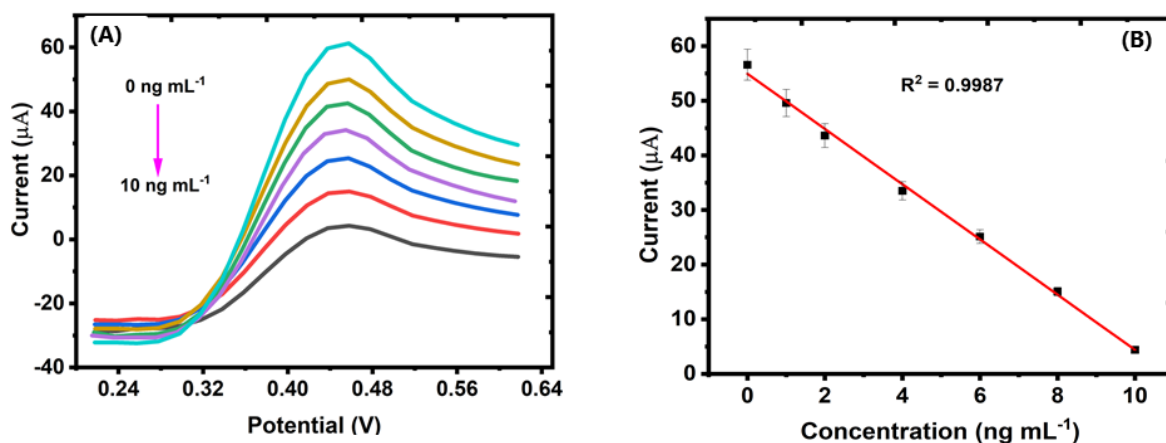


Fig. 5: (A) LSV curves for BSA/anti-AFB₁/L-Cys/PdNP-BN/CF when using different AFB₁ concentrations (0.0, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 ng mL⁻¹), and (B) Linear calibration curve of AFB₁ concentrations.

4.8. Recovery studies

The fabricated immunosensor capacity to identify AFB₁ in wine samples was then investigated by spiking wine samples with various AFB₁ concentrations chosen on the basis of the AFB₁ calibration curve (Fig. 5B). The fabricated immunosensor recovery levels varied between 93.00% and 106.00%, and the proportional error ranged from 1.83 to 6.0% (Table 1). These data indicate that the fabricated immunosensor can be used to detect AFB₁ in wine.

Table 1: Quantitative determination of AFB₁ in wine samples.

AFB ₁ added (ng mL ⁻¹)	AFB ₁ detected (ng mL ⁻¹)	Recovery (%)	Proportional error (%)
2.0	2.12	106.00	6.0
4.0	3.89	97.75	2.75
6.0	5.89	98.17	1.83
8.0	8.34	93.00	4.25

4.9. Computational modelling

4.9.1. Molecular docking

Molecular docking studies were performed to investigate the preferred binding sites of the BSA-anti-AFB₁ antibody substrate. **Fig. 6** shows the best docked pose and binding site of the substrate. The best binding site (site 1) was located in the hydrophobic cavity of BSA, known as subdomain IIA [76].

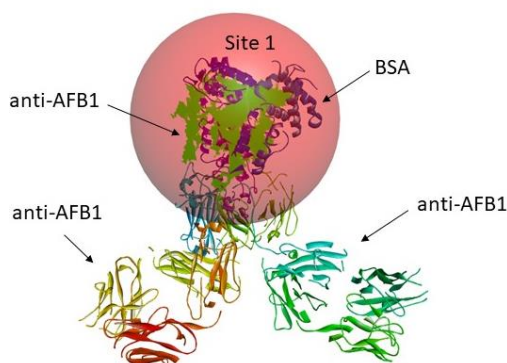


Fig. 6: Best docked pose and binding site for the BSA-anti-AFB₁ antibody complex.

4.9.2. Monte Carlo adsorption simulations

Monte Carlo adsorption simulations were performed to investigate the substrate-adsorbate interaction energetics and to validate the experimental (CV) results of the BSA/anti-AFB₁/L-Cys/PdNP-BN/CF immunosensor.

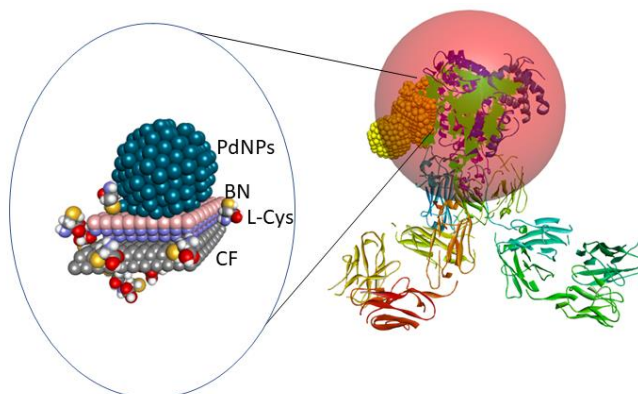


Fig. 7: Lowest energy configuration of the BSA/anti-AFB₁/L-Cys/PdNP-BN/CF immunosensor using Monte Carlo simulations.

The calculated adsorption energy for the immunosensor (**Fig. 7**) was strongly negative, indicating stabilization and an exothermic adsorption process [55, 57], implying stronger adsorption energy. This result explains why non-specific site blocking with BSA to form BSA-anti-AFB₁/L-Cys/PdNP-BN/CF resulted in a I_{pa} decrease to 0.50 μA (**Fig. 3A**, iv). The major forces that stabilized the complex were hydrogen bonding and van der Waals interactions.

Our modelling results (see supplementary information) also showed that the adsorption energies increased from the PdNP-BN/CF to the L-Cys/PdNP-BN/CF nanocomposite, suggesting higher stabilization due to the strong interaction of L-Cys with the electrode surface, in line with the amplified electrochemical signals in **Fig. 3A (i-ii)**. This also supports the catalytic behaviour of BN that facilitates electron transfer.

5. Conclusion

The anti-AFB₁ antibody was immobilized onto the L-Cys- and PdNP-functionalized BN/CF electrode for AFB₁ detection. The fabricated immunosensor displayed adequate reproducibility and high specificity and sensitivity for AFB₁ detection in wine samples, from 1.0 to 10 ng mL⁻¹ (LOD of 0.832 ng mL⁻¹). Our results demonstrated the multifunctional nanocomposite synergistic effects. Specifically, in the presence of EDC/NHS activation, L-Cys provided electron activity due to its electrochemical property, PdNPs offered sites for the antibody covalent immobilization, and BN gave good electric conductivity and rigidity to increase stability. Moreover, computational modelling was used to complement and validate the experimental data. The developed method offers a universal strategy for AFB₁ detection in wine, and could pave the way to the development of a sensitive device for wine safety and screening applications.

Declaration of Competing Interest

The authors state that they do not have any conflict of interest.

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