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Electrochemical Immunosensor Based on Polythionine/Gold Nanoparticles for the Determination of Aflatoxin B<sub>1</sub>

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Abstract: An aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) electrochemical immunosensor was developed by the immobilisation of aflatoxin B<sub>1</sub>-bovine serum albumin (AFB<sub>1</sub>-BSA) conjugate on a polythionine (PTH)/gold nanoparticles (AuNP)-modified glassy carbon electrode (GCE). The surface of the AFB<sub>1</sub>-BSA conjugate was covered with horseradish peroxidase (HRP), in order to prevent non-specific binding of the immunosensors with ions in the test solution. The AFB<sub>1</sub> immunosensor exhibited a quasi-reversible electrochemistry as indicated by a cyclic voltammetric (CV) peak separation (ΔE<sub>p</sub>) value of 62 mV. The experimental procedure for the detection of AFB<sub>1</sub> involved the setting up of a competition between free AFB<sub>1</sub> and the immobilised AFB<sub>1</sub>-BSA conjugate for the binding sites of free anti-aflatoxin B<sub>1</sub> (anti-AFB<sub>1</sub>) antibody. The immunosensor’s differential pulse voltammetry (DPV) responses (peak currents) decreased as the concentration of free AFB<sub>1</sub> increased within a dynamic linear range (DLR) of 0.6 - 2.4 ng/mL AFB<sub>1</sub> and a limit of detection (LOD) of 0.07 ng/mL AFB<sub>1</sub>. This immunosensing procedure eliminates the need for enzyme-labeled secondary antibodies normally used in conventional ELISA–based immunosensors.

Keywords: Immunosensor; Gold nanoparticles; Aflatoxin B<sub>1</sub>; Polythionine; Horseradish peroxidase (HRP).
1. Introduction

Aflatoxin B1 (AFB1) is an example of a group of highly toxic difurancoumarin derivatives that are produced by many strains of Aspergillus flavus and A. parasiticus which often contaminate a variety of food and animal feed stored under temperate and humid conditions favourable to mould growth [1]. The four major aflatoxins have been designated as B1, B2, G1 and G2 based on their fluorescence under UV light and their relative chromatographic mobility during thin layer chromatography. AFB1 has been classified as a group 1 human carcinogen and aflatoxins G1, G2 and B2 belong to a group human carcinogens [2]. These toxins exhibit carcinogenic, teratogenic and mutagenic properties and have now been isolated from a wide variety of agricultural products [3]. AFB1 can enter the food chain mainly through the ingestion of contaminated human or animal food. The intake of AFB1 over a long period of time, even in low concentrations, may be very deleterious to health [4]. The Food and Agricultural Organization 2004 report on mycotoxins [5] revealed that as of December 2003, at least 99 countries worldwide had regulations in place for permitted mycotoxin levels in food/feed, and have set limits for AFB1 alone or for the sum of aflatoxins B1, B2, G1 and G2. The maximum permissible level for AFB1 in food was set at 2 µg/kg (2 ppb).

Many analytical methods have been developed for the determination of aflatoxins. These include thin-layer chromatography (TLC) [6] and high-performance liquid chromatography (HPLC) [7]. Though these techniques have excellent sensitivities they typically require skilled operators, extensive sample pre-treatment and expensive equipment [8]. The goal of more recent studies has been to simplify and expedite the method of detection while attempting to maintain or improve the sensitivity. Among the immunochemical approaches, the enzyme-linked immunosorbent assay (ELISA) method is the most widely applied. Spectrophotometric ELISAs specific for AFB1 [9, 10], total aflatoxins [11, 12] and AFM1 [13, 14] have been developed and their simplicity, adaptability and sensitivity have been demonstrated. In order to achieve higher sensitivity and move to the use of disposable probes, electrochemical immunosensors for aflatoxins based on indirect competitive ELISA format have been proposed [15-17]. These immunosensors require the use of labeled secondary antibodies for detection. To achieve label-free immunosensors, direct electrochemical immunosensors for AFB1 based on electrochemical impedance spectroscopy [18], optical waveguide lightmode spectroscopy [19] and room temperature ionic liquids [20] have been reported.

The search for a simple and label-free amperometric immunosensor is of considerable interest. Among the various conducting polymers, thionine (phenothiazine) is a redox dye which has been studied extensively due to its potential utility in sensor applications [21, 22]. Its electroactivity lies not only in the heterocyclic nitrogen atoms and nitrogen bridges, but also in its free amine groups [23]. In addition polythionine (PTH) can be easily functionalised due to the abundant amino groups which adsorb metal ions and various organic halogen substances, thus preventing proteins from damage [24]. On the other hand, gold nanoparticles (AuNPs) have been extensively used as matrices for the immobilization of macromolecules such as proteins, enzymes and antibodies; as well as chemical labels for biomolecules [25-27]. Modification of electrode surfaces with AuNPs provides a microenvironment similar to what obtains under physiological conditions [28]. In this investigation, an electrochemical immunosensor for the detection of AFB1 was developed by the drop-coating of AuNPs on PTH-modified glassy carbon electrode (GCE) surface. Subsequently AFB1-conjugate was
adsorbed on to the gold nanoparticles surface. Details of the preparation, characterization and application of the immunosensor are described.

2. Experimental

2.1 Reagents and materials

Analytical reagent grade chemicals from Sigma-Aldrich were used in all experiments. Phosphate buffer saline (PBS) solution pH 7.2 contained 0.1 M KH₂PO₄, 0.1 M Na₂HPO₄, 2.7 mM KCl and 0.137 M NaCl. Acetate buffer (pH 6.5) was prepared from 0.1 M CH₃COONa, 0.1 M CH₃COOH and 0.1 M KCl. 1 mg/mL Aflatoxin B₁ (AFB₁) solution was prepared by dissolving AFB₁ from Aspergillus flavus in methanol followed by dilution in PBS/10% methanol to give a series of standard solutions with a concentration range of 0.1-3.0 ng/mL. The antibody reagent was an immunoglobin (Ig) fraction of rabbit antiseraum AFB₁ (anti-AFB₁) antibody that contained 6.8 mg/mL of total protein (which has reactivity with aflatoxins B₁, G₁ and B₂, but no cross reactivity with B₂a, G₂, G₂a or M₁) and 0.15 M NaN₃ as preservative. The anti-AFB₁ antibody solution for immunosensing reactions was prepared by diluting the stock 6.8 mg/mL solution to 1:2000 v/v in PBS and stored at -20 °C when not in use. Aflatoxin B₁-BSA conjugate (8–12 mol AFB₁ per mol BSA), horseradish peroxidase (HRP; 169 units/mL lyophilised powder; EC.1.11.1.7; M₆ 4000), thionine acetate (3,7-diamino-5-phenothiazinium acetate; C₁₂H₉N₃S.C₂H₄O₂; M₆ 287.34), tetrachloro-auric acid (HAuCl₄.3H₂O; M₆ 393.83), sodium citrate and 30% hydrogen peroxide solution were other reagents used in the studies. Colloidal gold nanoparticles (AuNP) (diameter = 20 nm) were prepared according to the procedure of Yuan et al., 2004 [29] by adding 2 mL of 1% (w/w) sodium citrate to a boiling solution of 50 mL 0.01% (w/w) tetrachloro-auric acid. The AuNP solution was stored in a refrigerator in a dark-colored glass bottle. The production of colloidal AuNP was confirmed by UV-Vis measurement covering 200 – 700 nm at room temperature using distilled water as the reference, which gave a maximum absorption at 520 nm. The particle size of the AuNP was determined with transmission electron microscopy (TEM), in which a sample of the colloidal AuNP was dropped on a carbon-coated copper grid and left to dry for 24 h, after which TEM images were recorded and analysed.

2.2 Instrumentation

All electrochemical experiments were performed with a BAS/50W integrated automated electrochemical workstation (Bioanalytical Systems Lafayette, IN, USA). Cyclic voltammetry and differential pulse voltammetry experiments were performed with Ag/AgCl (3 M NaCl type) and platinum wire as reference and auxiliary electrodes, respectively. A BAS 3-mm diameter GCE in bare or modified form, was used as the working electrode. Transmission electron microscopy studies were carried out with JEOL JEM-1200 EX II electron microscope. UV-Vis experiments were performed with GBC UV/Vis 920 spectrophotometer (GBC Scientific Instruments, Australia).
2.3 Immunosensor preparation

Before use, the GCE was polished consecutively with 1.0, 0.3 and 0.05 micron aqueous slurry of alumina micropolish (Buehler, IL, USA), followed by sonication in double-distilled water and ethanol for 5 min and dried in air. 0.1 mM thionine was polymerised on the GCE by cyclic voltammetry (CV) in a 20-voltammetric cycle experiment covering a potential window of -400 to +1200 mV at a scan rate of 50 mV/s and a sensitivity of 0.001 A/V to produce the required PTH. Freshly prepared PTH film was drop-coated with 2 µL of colloidal AuNP and allowed to dry for 24 h. Subsequently, the electrode was coated with 5 µL of AFB1-BSA conjugate (1 µg/mL) and incubated for 1 h at 37 °C. The resultant immunosensor was incubated in 1 mg/mL HRP contained in acetate buffer (pH 6.5) for 60 min at 4 °C in order to block any remaining active sites of the AuNP layer and avoid non-specific adsorptions. The immunosensor was stored at 4 °C when not in use. The schematic illustration of the stepwise immunosensor assembly procedure is shown in Scheme 1.

Scheme 1. Reaction scheme for the preparation of aflatoxin B1 immunosensor. Step 1: polymerization of thionine. Step 2: formation of AuNP layer. Step 3: loading of AFB1-BSA conjugate. Step 4: blocking of AFB1-BSA conjugate layer with HRP.

2.4 Procedure for electrochemical immunosensing

Competitive immunosensing was performed in the absence and presence of free AFB1. Experiment in the absence of free AFB1 (i.e. 0.0 ng/mL AFB1) were performed by placing 5 µL of anti-AFB1 antibody on top of the immunosensor (i.e. GCE|AuNP|PTH|AFB1-BSA-conjugate HRP) and allowed to react for 15 min. The electrode system was immersed in a 1 mL cell solution containing acetate buffer pH 6.5 and 3.2 µM H2O2 and DPV measurement was performed by scanning cathodically from 0 to -480 mV at 10 mV pulse amplitude, 50 ms pulse width and 20 mV/s potential scan rate. For experiments in the presence of free AFB1 (i.e. 0.6 – 3.0 ng/mL AFB1), 10 µL of anti-AFB1 antibody
solution was mixed with 10 µL of 0.6, 1.2, 1.8, 2.4 or 3.0 ng/mL AFB₁ solutions. 5 µL of this mixture was placed on top of the immunosensor and allowed to react for 15 min. This was followed by DPV measurement as described above for 0.0 ng/mL AFB₁.

3. Results and Discussion

3.1 Characterisation of gold nanoparticles

UV-Vis spectrum of the colloidal gold nano-particles prepared by the method of Yuan et al. [29] showed a maximum absorption, $\lambda_{\text{max}}$, at 520 nm (Figure 1). The formation of nano-particles and the particle size (20 nm diameter) were confirmed by the TEM data (Figure 2). These results agreed with what has been reported for colloidal AuNP [29].

Figure 1. UV-Vis spectrum of colloidal gold nanoparticles.

Figure 2. TEM image of colloidal gold nanoparticles.
3.2 Electropolymerization of multiporous thionine film

The GCE was first reduced in acetate buffer solution (pH 6.5) containing thionine at -1500 mV to make it negatively charged and interact with positively charged thionine in the mildly acidic conditions. Subsequently cyclic voltammetry was performed at a bias voltage of -400 to +1200 mV at a scan rate of 50 mV/s. When the applied potential exceeded +1100 mV, the electropolymerization reaction proceeded and formed cationic radical species on the GCE [30]. This process resulted in a multiporous structure which facilitated the assembly of AuNP. The oxidation potential in the first step was the most important factor for electropolymerization of thionine and should not be less than +1100 mV. Two reasons suffice for this. Firstly in order to achieve the formation of polythionine (PTH) film, the electrode potential must be larger than the potential at which the oxidations of -NH$_2$ groups of thionine molecule occurs, and secondly the modified cation must be necessarily associated with the surface activation of GCE. During thionine electropolymerization, a pair of quasi reversible redox peaks with a cathodic peak potential ($E_{pc}$) of -250 mV and an anodic peak potential $E_{pa}$ of -100 mV, increased gradually and tended to become stable with increasing number of polymerization cycles (Figure 3a). On removal of the electrode from the dye-containing solution, a golden film was seen on the electrode surface. The CV of the GCE|PTH in acetate buffer (pH 6.5) (Figure 3b) confirmed the presence of surface-bound electroactive material. Figure 4 shows the scan rate dependence of the electrochemistry of GCE|PTH and GCE|PTH|AuNP|AFB$_1$-BSA-conjugate electrodes in acetate buffer pH 6.5. The peak currents varied linearly with scan rate as is characteristic of the electrochemistry of a surface-bound thin film of electroactive material [31]. It is thus suggested that nearly all the reduced polythionine (PTH$_{\text{red}}$) was converted to the oxidized polythionine (PTH$_{\text{ox}}$) on the forward scan and vice versa.

Figure 3. Cyclic voltammograms (a) for the synthesis of PTH from 0.1 mM thionine in acetate buffer (pH 6.5); and (b) of GCE|PTH in acetate buffer (pH 6.5). (Scan rate: 50 mV/s)
3.3 Electrochemical characteristics of the electrode

The stepwise assembly of the immunosensor was monitored cyclic-voltammetrically in acetate buffer pH 6.5 and the results are shown in Figure 5. The CV of the bare GCE is shown as Figure 5a. The bare GCE did not exhibit any redox chemistry over the potential window (-500 to 0 mV) used in the study. The GCE|PTH gave a quasi reversible electrochemistry with a peak separation of ~62 mV at 10 mV/s (Figure 5b). The redox couple can be ascribed to the electrochemistry of the PTH film on the GCE. The peak currents increased after the modification of GCE|PTH with AuNP (Figure 5e). The reason for this increase is that nanometer-sized particles of colloidal gold behave like a conducting wire or electron conducting tunnel, which makes it easier for the electron transfer to take place. However, the peak currents decreased (Figure 5d) after AFB$_1$-BSA conjugate was incorporated to
produce GCE|PTH|AuNP|AFB1-BSA-conjugate, which indicated that the relatively insulating AFB1-BSA conjugate was immobilized on the electrode surface. The subsequent blocking of possible active sites with HRP to form GCE|PTH|AuNP|AFB1-BSA-conjugate|HRP immunosensor system decreased the CV peak current due to the adsorption of the protein, HRP (Figure 5c). Surface concentration (Γ*) values of $7.06 \times 10^{-11}$ and $9.65 \times 10^{-10}$ mol/cm² were calculated for GCE|PTH and GCE|PTH|AuNP|AFB1-BSA-conjugate electrode systems, respectively.

**Figure 5.** CV’s of (a) bare GCE; (b) GCE|PTH; (c) GCE|AuNP|PTH|AFB1-BSA-conjugate|HRP-blocked; (d) GCE|AuNP|PTH|AFB1-BSA-conjugate and (e) GCE|AuNP|PTH. Conditions: acetate buffer ph 6.5; scan rate = 10 mV/s.

3.4 Assay of the HRP enzymatic catalytic activity

Horseradish peroxidase was used in the preparation of the immunosensors to block possible uncovered active sites on the GCE|AuNP|PTH|AFB1-BSA-conjugate in order to avoid non-specific adsorption. Experiments were performed with hydrogen peroxide to ascertain if HRP was indeed immobilized on the electrode. Figure 6 depicts the CV’s of the proposed immunosensor in the presence and absence of H₂O₂. A redox couple representing the electrochemistry of PTH was observed in the absence of H₂O₂ (Figure 6a). However, an enhancement of the cathodic peak and a concomitant decrease of the anodic peak current were observed in the presence of 3.2 µM H₂O₂ (Figure 6b). It can thus be concluded that HRP was attached to the immunosensor surface and it retained its enzymatic catalytic activity [21], which is coupled to the PTH electron transfer process shown below.
The optimal amount of H$_2$O$_2$ required for the immunosensing reaction with GCE|AuNP|PTH|AFB$_1$-BSA-conjugate|HRP-blocked electrode system was determined from steady-state amperometry experiments performed at -175 mV. As shown in Figure 7, the sensor gave a maximum response at 3.2 µM H$_2$O$_2$. At H$_2$O$_2$ concentrations higher than 3.2 µM, the sensor response decreased owing to the irreversible transition of the immobilized HRP to its inactive form [32]. As a result, 3.2 µM H$_2$O$_2$ was chosen for the immunosensing experiments.

**Figure 6.** CV’s of AFB$_1$ immunosensor (GCE|AuNP|PTH|AFB$_1$-BSA-conjugate|HRP-blocked) in acetate buffer (pH 6.5): in the absence (a), and presence (b), of 3.2 µM H$_2$O$_2$. Scan rate = 10 mV/s.

![Figure 6](image1)

**Figure 7.** Steady-state amperometric responses of AFB$_1$ immunosensor (GCE|AuNP|PTH|AFB$_1$-BSA-conjugate|HRP-blocked) to H$_2$O$_2$ in acetate buffer (pH 6.5) at -175 mV. Inset: H$_2$O$_2$ calibration curve obtained from the steady state responses.

![Figure 7](image2)

The effect of pH on the immunosensor was studied for pH’s 4.5 to 6.5 at 25 °C. In order to retain the bioactivity of both AFB$_1$-BSA conjugate and HRP, pH 6.5 was chosen as the optimal pH. Although 37 °C is the best incubation temperature for antibody-antigen reactions, immunoproteins and HRP do not maintain their activities for a long time at this temperature. As a result 25 °C was used in all experiments.
3.4 Performance of the immunosensor

The responses of the immunosensor to AFB$_1$ were recorded with DPV as shown in Figure 8 for 0 – 3 ng/mL AFB$_1$. As expected, the peak current was inversely proportional to the analyte concentration. The detection principle is based on the inhibition of the active centre of HRP by the formation of antigen-antibody complex. The observed current is attributed to the catalytic response of the adsorbed HRP to H$_2$O$_2$. The formation of the antigen-antibody complex introduces a local current change at the sites of the adsorbed HRP due to the inhibition of the H$_2$O$_2$ reduction reaction. The attenuation of the DPV response is dependent on AFB$_1$ concentration as shown in Figure 8 and it is attributable to the increase in electron transfer resistance of adsorbed HRP [33] caused by the insulating properties of the complex formed by the binding of AFB$_1$-BSA conjugate and anti-AFB$_1$ antibody.

**Figure 8.** DPV responses of AFB$_1$ immunosensor (GCE|AuNP|PTH|AFB$_1$-BSA-conjugate|HRP-blocked), for 0 - 3 ng/mL AFB$_1$ (mixed with anti-AFB$_1$ antibody) in acetate buffer (pH 6.5) containing 3.2 µM of H$_2$O$_2$. DPV experimental conditions: scan rate = 20 mV/s; pulse amplitude = 10 mV; and pulse width = 50 ms.

![Figure 8](image)

**Figure 9.** Calibration plot of AFB$_1$ immunosensor. Conditions are as given in Figure 8.

![Figure 9](image)
The calibration graph of the AFB$_1$ immunosensor (Figure 9) plotted from DPV results gave DLR, sensitivity and LOD values of 0.6 - 2.4 ng/mL, 1.23 x 10$^{-6}$ A/(ng/mL) and 0.07 ng/mL, respectively. The DPV measurements were carried out in triplicates. The relative standard deviations of the DPV responses were 3.1%, 2.9%, 2.4% and 5.1% for 0.6, 1.2, 1.8 and 2.4 ng/mL, respectively, indicating acceptable level of precision.

Table 1. Comparison of the analytical parameters of AFB$_1$ immunosensor.

| Immunosensor                                      | DLR (ng/mL) | LOD (ng/mL) | Reference |
|--------------------------------------------------|-------------|-------------|-----------|
| 96-well screen printed microplate                | 0.05 – 2    | 0.03        | [15]      |
| Pt|PSSA|PANi|Anti-AFB$_1$ | 0.1 - 0.6   | 0.1        | [18]      |
| GCE|Nafion|RTIL|TiO$_2$|AuNP|Anti-AFB$_1$-HRP | 0.1 – 12 | 0.05 | [20] |
| GCE|AuNP|PTH|AFB$_1$-BSA-conjugate|HRP-blocked | 0.6 - 2.4 | 0.07 | This work |

4. Conclusions

The DLR and LOD of the immunosensor were compared with those of other electrochemical AFB$_1$ immunosensors [15, 18, 20] as shown in Table 1. The values of these sensor parameters for the PTH-based immunosensor are in good agreement with those reported by other laboratories. In addition, the sensor exhibited high sensitivity and good reproducibility. These characteristics of the immunosensor show that it can be used to screen food products for AFB$_1$, since the DLR and LOD values cover the FAO’s limit of 2 ppb AFB$_1$ in food samples [5]. The immunosensing procedure reported in this study eliminated the requirement of secondary labeled antibodies as is the case in conventional electrochemical immunosensors based on ELISA techniques.

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References

1. Cole, R.J.; Cox, R.H. Handbook of Toxic fungal metabolites. Academic Press: New York, 1981.
2. International Agency for Research on Cancer. IARC Monographs on the evaluations of Carcinogenic Risks to Humans. IARC: Lyon, 1993; vol. 56, pp. 489-521.
3. Moss, O.M. Risk assessment for aflatoxins in foodstuffs. Int. Biodeterior. Biodegrad. 2002, 50, 137-142.
4. Miraglia, M.; Brera, C.; Colatosti, M. Application of Biomarkers to Assessment of Risk to Human Health from Exposure to Mycotoxins. Microchem. J. 1996, 54, 472-477.
5. Van Egmond, H.P.; Jonker, A.R.O. Worldwide regulations for mycotoxins in food and feed in 2003. FAO Food and Nutrition paper 81: Report of the Food and Agriculture Organization of the United Nations: Rome, 2004.
6. Fernandez, A.; Belio, R.; Ramos, J. J.; Sanz, M.C.; Saez, T. Aflatoxins and their metabolites in the tissues, faeces and urine from lambs feeding on an aflatoxin-contaminated diet. *J. Sci. Food Agric.* 1997, 74, 161-168.

7. Jaimez, J.; Fente, C.A.; Vazquez, B.I.; Franco, C.M.; Capeda, A.; Mahhuzier, G.; Prognon, P. Application of the assay of aflatoxins by liquid chromatography with fluorescence detection in food analysis. *J. Chromatogr. A.* 2000, 882, 1-10.

8. Stroka, J.; Anklam, E. New strategies for the screening and determination of aflatoxins and the detection of aflatoxin-producing moulds in food and feed. *Trends. Anal. Chem.* 2002, 21, 90-95.

9. Dutta, T.K.; Das, P. Isolation of aflatoxigenic strains of *Aspergillus* and detection of aflatoxin B₁ from feeds in India. *Mycopathologia* 2000, 151, 29-33.

10. Kolosova, A.Y.; Shim, W.B.; Yang, Z.Y.; Eremin, S.A.; Chung, D. H. Direct competitive ELISA based on a monoclonal antibody for detection of aflatoxin B1. Stabilization of ELISA kit components and application to grain samples. *Anal. Bioanal. Chem.* 2006, 384, 286-294.

11. Ayciek, H.; Aksoy, A.; Saygi, S. Determination of aflatoxin levels in some dairy and food products consumed in Ankara, Turkey. *Food Control.* 2005, 16, 263-266.

12. Zheng, H.; Humphney, C. W.; King, R.S.; Richard, J.L. A review of rapid methods for the analysis of aflatoxins. *Mycopathologia.* 2005, 159, 1-9.

13. Yaroglu, T.; Oruc, H.H.; Tayar, M. Aflatoxin M₁ levels in cheese samples from some provinces of Turkey. *Food Control.* 2005, 16, 883-885.

14. Rastogi, S.; Divedi, P.D.; Khanna, S.K.; Das, M. Detection of Aflatoxin M₁ contamination in milk and infant milk products from Indian markets by ELISA. *Food Control.* 2004, 15, 287-290.

15. Piermarini, S.; Micheli, L.; Ammida, N.H.S.; Palleschi, G.; Moscone, D. Electrochemical immunosensor array using a 96-well screen-printed microplate for aflatoxin B₁ detection. *Biosens. Bioelectron.* 2007, 22, 1434-1440.

16. Micheli, L.; Grecco, R.; Badea, M.; Moscone, D.; Palleschi, G. An electrochemical immunosensor for aflatoxin M₁ determination in milk using screen-printed electrodes. *Biosens. Bioelectron.* 2005, 21, 588-596.

17. Pemberton, R.M.; Pittson, R.; Biddle, N.; Drago, G.A.; Hart, J.P. Studies towards the development of a screen-printed carbon electrochemical immunosensor array for mycotoxins: A sensor for Aflatoxin B₁. *Anal. Lett.* 2006, 39, 1573-1586.

18. Owino, J. H. O.; Ignaszak, A.; Al-Ahmed, A.; Baker, P.G. L.; Alenu, H.; Ngila, J.C.; Iwuoha, E. I. Modelling of the impedimetric responses of an aflatoxin B₁ immunosensor prepared on an electrosynthetic polyaniline platform. *Anal. Bioanal. Chem.* 2007, 388, 1069-1074.

19. Adanyi, N.; Levkovets, I. A.; Rodriguez-Gil, S.; Ronald, A.; Varadi, M.; Szendro, I. Development of immunosensor based on OWLS technique for determining Aflatoxin B1 and Ochratoxin A. *Biosens. Bioelectron.* 2007, 22, 797-802.

20. Sun, A.; Qi, Q.; Dong, Z. L.; Liang, K.Z. An electrochemical enzyme immunoassay for aflatoxin B1 based on bio-electrocatalytic reaction with room-temperature ionic liquid and nanoparticle-modified electrodes. *Sens. & Instrumen. Food Qual.* 2008, 2, 43-50.

21. Ruan, C.; Yang, F.; Lei, C.H.; Deng, J.Q. Thionine covalently tethered to multilayer horseradish peroxidise in a self assembled monolayer as an electron transfer mediator. *Anal. Chem.* 1998, 70, 1721-1725.
22. Xiao, Y.; Ju, H.; Chen, H.Y. A reagentless hydrogen peroxide sensor based on incorporation of horseradish peroxidase in poly(thionine) film on a monolayer modified electrode., *Anal.Chim Acta.* **1999**, *391*, 299-306.

23. Reid, G.D.; Whittaker, D.J.; Day, M.A.; Creely, C.M.; Tuite, E.M.; Kelly, Beddard, G.S. Ultrafast Electron-Transfer Reactions between Thionine and Guanosine Bases. *J. Am.Chem. Soc.* **2001**, *123*, 6953-6954.

24. Dohno, C, Stemp, E.D.A.; Barton, J. K. Fast Back Electron Transfer Prevents Guanine Damage by Photoexcited Thionine Bound to DNA. *J. Am.Chem. Soc.* **2003**, *125*, 9586-9587.

25. Storhoff, J.J.; Elghanian, R.; Music, R.C.; Markin, C.A.; Lestinger, R.L. One pot colorimetric differentiation of polynucleotides with single base imperfections using gold nanoparticles. *J. Am.Chem. Soc.* **1998**, *120*, 1959-1964.

26. Tang, D.P.; Yuan, R.; Chai, Y.Q.; Dai, J.Y.; Zhong, X.; Liu, Y. A novel immnosensor based on immobilization of hepatitis B surface antibody on platinum electrode modified colloidal gold and polyvinyl butyral as matrices via electrochemical impedance spectroscopy. *Bioelectrochem.* **2004**, *65*, 15-22.

27. Xu, S.Y.; Han, X.Z. A novel method to construct a third generation biosensor: self assembling gold nanoparticles on thiol-functionalized polystyrene-co-acrylic acid) nanospheres. *Biosens. Bioelectron.* **2004**, *19*, 1117-1120.

28. Liu, S.Q.; Leech, D.; Ju, H.X. Application of colloidal gold in protein immobilization, electron transfer and biosensing. *Anal. Lett.* **2003**, *36*, 1-17.

29. Yuan, R.; Tang, D.; Chai, Y.; Zhong, X.; Liu, Y.; Dai. Ultrasensitive potentiometric immnosensor based on SA and OCA techniques for immobilization of HBsAb with colloidal Au and Polyvinyl Butyral as matrixes. *Langmuir.* **2004**, *20*, 7240-7245.

30. Yang, R.; Ruan, C.; Dai, W.; Deng, J.; Kong, J. Electropolymerization of thionine in neutral aqueous media and H2O2 biosensor based on poly(thionine). *Electrochim. Acta.* **1999**, *44*, 1585-1596.

31. Murray, R.W. In: Bard, A.J. (ed), Electroanalytical chemistry. Marcel Dekker: New York, 1984; vol. 13, p.191.

32. Ju, H.X.; Yan, G.F.; Chen, H.Y. Enzyme-Linked Immunoassay of α-1-Fetoprotein in Serum by Differential Pulse Voltammetry. *Electroanalysis.* **1999**, *11*, 124-129.

33. Lu, X; Bai, H; He, P; Cha, Y; Yang, G; Tan, L; Yang, Y.A reagentless amperometric immnosensor for α-1-fetoprotein based on gold nanowires and ZnO nanorods modified electrode. *Anal.Chim.Acta.* **2008**, *615*, 158-164.

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