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CdS NANOPARTICLE-BASED BIOSENSOR DEVELOPMENT FOR AFLATOXIN DETERMINATION

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ABSTRACT

An anodic stripping voltammetry detection method with lateral flow immunochromatographic assay for determining aflatoxin M_1 was developed. Cadmium sulfide nanoparticles (CdSNPs) were employed as a label for aflatoxin antibody. Conjugation between the CdSNPs and aflatoxin antibody was performed as the first step. The CdSNP-antibody conjugate was then immobilized on a test strip on a conjugate pad. When the conjugate interacted with the sample containing aflatoxin, it was captured by the aflatoxin in the test zone. The CdSNPs retained in the test zone were then measured by anodic stripping voltammetry using a boron-doped diamond electrode. A linear concentration range of 0–70 ppb aflatoxin, with a sensitivity of 20 µA/mM and a detection limit of 30 ppb, was achieved by this method.

Keywords: Aflatoxin; Anodic stripping voltammetry; Boron-doped diamond; CdS nanoparticles

1. INTRODUCTION

Aflatoxin is a secondary metabolite, mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, which contaminate agricultural products, namely nuts, cereals and animal feeds (Bennett & Klich, 2003). Six important types of aflatoxin have been identified and are denoted as B_1, B_2, G_1, G_2, M_1 and M_2 (Wacoo et al., 2014). Among these types, the International Agency for Research on Cancer classified aflatoxin B_1 (AFB_1) as a group 1 carcinogenic substance due to its ability to disrupt the induction of certain enzymes and inhibit RNA synthesis (IARC, 2002). Meanwhile, aflatoxin M_1 (AFM_1) is a hydrolyzed metabolite of AFB_1, produced by the mammary gland; hence, it is mainly found in milk and dairy products, such as cheese and yoghurt (Martins & Martins, 2000). Due to its stability during pasteurization and other heat treatment (Wang et al., 2009), as well as its hepatotoxicity and carcinogenic effects (Badea et al., 2004), the aflatoxin content must be controlled. The regulated upper limits for AFB_1 and total aflatoxins (B_1, B_2, G_1 and G_2) are 30 and 50 µg/kg respectively, while that for AFM_1 in milk is 1.0 µg/kg. (IARC, 2002).

In recent years, many analytical techniques for quantitative aflatoxin determination have been studied, including thin layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC) (Roseanu et al., 2010). These techniques are sufficiently sensitive for aflatoxin; however, GC and HPLC operation is time-consuming and

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requires a skilled analyst, complex sample pre-treatment and expensive instrumentation, which affect aflatoxin detection in food products. Beside TLC, another fast detection method is immunochemistry. This can be used to detect aflatoxin in solid food (Betina, 1985; Shepard, 2009) as well as dairy products (Anfossi et al., 2008; Li et al., 2009). Immunochemistry has advantages with respect to its selectivity and sensitivity. One immunochemistry development is lateral flow immunoassay. Furthermore, enzyme-linked immunosorbent assay, which depends on a specific interaction between an antigen toxin target and its antibody, is also widely used to detect aflatoxin (Zhang et al., 2016). However, this method has several drawbacks, such as the requirement of an incubation period, together with time-consuming washing and mixing (Badea et al., 2004). Accordingly, the development of a new strategy for aflatoxin determination is urgently required.

To achieve a more suitable aflatoxin detection technique, several fast methods based on biosensors or immunosensor applications have recently been developed, including flow-injection immunoassay (Badea et al., 2004); a microcomb electrode modified with a self-assembly horseradish peroxidase (HRP) and anti-AFB_1 (Liu et al., 2006); and electrochemical methods (Piermarini et al., 2007; Parker & Tothill, 2009; Linting et al., 2012; Chauhan et al., 2015; Zhang et al., 2016; Gouda et al., 2016; Kong et al., 2018; Peng et al., 2018). Additionally, Paniel et al. (2010) developed an electrochemical immunosensor for AFM_1 in food products based on a competitive immunoassay with HRP as the label. A fast and simple immunochromatography test was also developed to detect AFB_1 in less than 10 min. The developed AFB_1 sensor test also shows no cross-reaction with ochratoxin A. (Moon et al., 2012).

Electrochemical measurements were have been performed by anodic stripping voltammetry (ASV) using a boron-doped diamond (BDD) as the working electrode. ASV is generally considered to be the most suitable method for trace metal detection, since this offers certain advantages for metal analysis, including excellent selectivity and sensitivity, a low detection limit, simple operation and a relatively low-cost process (Ivandini et al., 2012). BDD is superior to other conventional solid electrodes due to its wide potential window, low capacitance, chemical inertness, mechanical durability and exceptional biocompatibility (Fujishima et al., 2005, Ivandini & Einaga, 2017). The use of the ASV technique for immunochromatographic test strips using BDD electrodes has been reported (Wicaksono et al., 2014; Ivandini et al., 2015; Hayat et al., 2016).

The above methods mainly yield only qualitative results. The electrochemical method offers accurate quantitative results but requires coupling with another relatively complex method. In this research, a method for determining aflatoxin was developed which combines the speed and reproducibility of the immunochromatography technique with the excellent selectivity and sensitivity of the electrochemical method. As a label, cadmium sulfide nanoparticles (CdSNPs) were used. These are suitable as labels in immunochromatographic sensors, since they are inexpensive and exhibit low potential oxidation, good electrical conductivity (Zheng et al., 2015), and high sensitivity (Du et al., 2012). Although no research has been conducted on the use of CdS as an aflatoxin label, CdSNPs have been investigated as labels for several biosensors, such as a cyanide ion sensor (Salariya et al., 2017), DNA (Svitkova et al., 2017), and glucose (Huang et al., 2005). Nanoparticles can be prepared by several methods, including the microwave technique (Usman et al., 2018), the hydrothermal method (Fadli et al., 2017), the alkaline hydrolysis method (Khalil et al., 2018), and microemulsions (Mirgorod & Efimova, 2007). The advantages of the synthesis of nanoparticles in microemulsions are that the process occurs in mild conditions, and that the size and shape of the nanoparticles can be controlled by the micelle structure and dynamics (Mirgorod & Efimova, 2007).

2. METHODOLOGY

2.1. Material

Aflatoxin from *A. flavus* was supplied by Sigma Aldrich, USA. The aflatoxin antibody (anti-AFM_1) was made in our laboratory (Assaat et al., 2018) and purified as described by Prihantoro et al. (2017). Nitrocellulose membrane, Tween® 20, sucrose, sodium azide, $Cd(NO_3)_2$, Na_2S, sodium dioctyl sulphosuccinate (SDOSS) as surfactant, isooctane and other chemicals were obtained from Wako, Japan. All the chemicals were used without further purification. Pure water was obtained from a simple water system (Direct-Q UV 3-Millipore).

2.2. Electrode Preparation

The BDD electrode was prepared using microwave plasma-assisted chemical vapor deposition (MPACVD, ASTeX Corp., USA) with a mixture of 50 mL acetone and 4 mL trimethoxy borane as the precursor. The preparation was performed according to Fujishima et al. (2005). A particle size of 5 µm was obtained with a film thickness of around 8 µm. The characterization by Raman spectroscopy (Renishaw System 2000, UK) generated a typical peak at 1333 cm^{-1} corresponding to the ansp^3 carbon bond, and two peaks at 500 and 1200 cm^{-1}confirmed the presence of boron in the diamond structure (Ushizawa et al., 1998; Fujishima et al., 2005; Honda et al., 2013).

2.3 CdS Nanoparticle and CdSNP-Anti Aflatoxin Conjugate Preparation

The CdSNPs were prepared by the microemulsion method, in line with Mirgorod and Efimova (2007). Briefly, a mixture of water, 3.55 g sodium dioctyl sulphosuccinate and 60.5 g isooctane was divided into two parts (31.7 mL each). Then, 1 mL of 0.1 M $Cd(NO_3)_2$ solution was added to one part, while 1 mL of 0.1 M Na_2S was added to the other. The suspension was thoroughly stirred to completely disperse both solutions, followed by sonication for 5 min. Subsequently, mixing and stirring were performed for several more seconds until a faint yellow colour appeared, indicating the formation of CdS particles. The solutions were evaporated to reduce their volume and a mixture of 3-mercaptopropionic acid (20 mg mL^{-1}, 40.6 µL) and sodium 3-mercapto-1-propanesulphonate (20 mg mL^{-1}, 68.16 µL) was added. Precipitation was performed in a dark chamber at 23°C for 12 h. The nanoparticles were subsequently washed with pyridine, hexane, ethyl acetate, ethanol and pure water to obtain a yellow CdS suspension. The washed precipitate was stored in 5 mL pH 6 phosphate buffer solution (PBS) for further use.

The CdSNP–anti-aflatoxin conjugate was prepared according to a procedure conducted by Valera et al., (2012). The obtained CdSNPs were added to 5 mM PBS (437.5 µL, pH 8.0). 125 µL of this suspension was then added to 1.0 mL 5 mM PBS containing 20 mM 1-ethyl-3,3-dimethyl aminopropyl carbo diimide (EDC) solution and 10 mM N-hydroxy succinimide (NHS), before stirring for 15 min at medium speed. The mixture was centrifuged, and the supernatant was isolated to remove excess reagent. The activated CdSNPs were then added to 2.0 mL aflatoxin antibody solution (1 mg mL^{-1}), which had been previously dissolved in 0.5 mL 10 mM PBS (pH 7.4). The solution was then stirred for 12 h at 37°C to form a covalent bond between the activated CdSNP and aflatoxin antibody. The excess unreacted carboxyl group was blocked with glycine amino acid (10 µL 10 mg mL^{-1}) and re-stirred for 5 h. The CdSNP–anti-aflatoxin conjugate was then suspended in 1 mL PBS (pH 8.0) to obtain 3.5 mL of 0.6 mg/mL CdSNP–anti-aflatoxin conjugate, which was stored at 4°C for further use.

2.4. Test Strip Fabrication

The test strips for the aflatoxin biosensors comprised a sample pad, a conjugate pad, a test line, a control line, an absorbent pad and a nitrocellulose membrane. All the components were arranged on a plastic sheet for support, as shown in Figure 1.

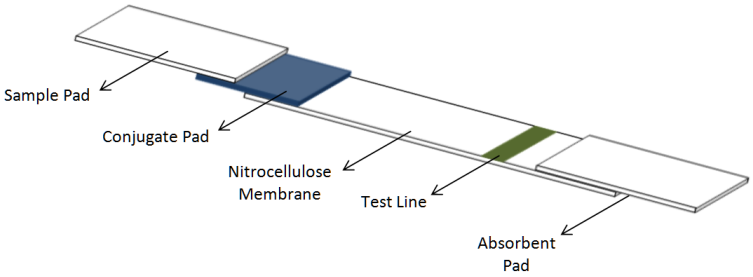


Figure 1 Scheme of immunochromatographic strip test assay

The sample pad was prepared by dropping the borate buffer solution containing bovine serum albumin (BSA) and Tween-20 onto a fibreglass pad, while the conjugate pad was prepared by dropping the same buffer solution containing BSA, Tween-20, sucrose and sodium azide onto conjugate pad. The pads were dried at 37°C over night. The nitrocellulose membrane was soaked in PBS and 150 µL CdSNP–anti-aflatoxin conjugate was then dropped onto the conjugate pad and redried.

2.5. ASV Measurements

ASV measurements were made in an electrochemical cell with a three-electrode system. BDD was used as the working electrode, while Pt wire and Ag/AgCl were employed as the counter and reference electrodes respectively. A 0.1 M $HClO_4$ solution was used as the electrolyte. Prior to the ASV measurement of the CdSNPs, the ASV measurement parameters, including the deposition potential, deposition time and scan rate were optimised for Cd^{2+} ions. ASV measurements of the test strips were then made based on the optimization results.

Aflatoxin detection using a CdS-based biosensor was achieved based on the competition between the aflatoxin immobilized on a test zone and the free aflatoxin contained in the tested sample. The measurement was made for a standard solution of aflatoxin (0–70 ppb) prepared by dilution in methanol and a PBS mixed solution. During the measurements, the sample pad was soaked in each standard solution for ~7 min. Theoretically, the sample will move along the nitrocellulose membrane through the conjugate pad to the test line. Furthermore, the test zone was cut and dissolved in 4 mL 0.1 M $HClO_4$ solution and left to react for 5min. Subsequently, electrochemical measurement was conducted by ASV.

3. RESULTS AND DISCUSSION

3.1. CdS Nanoparticle and CdS-NP – Anti Aflatoxin Conjugate Preparation

CdSNPs were prepared by the micro emulsion method, using $Cd(NO_3)_2$ as the Cd^{2+} source and Na_2S as the S^{2-} source, according to the following equation:

$$Cd(NO_3)_{2(aq)} + Na_2S_{(aq)} \rightarrow CdS_{(s)} + 2Na(NO_3)_{(aq)} \quad (1)$$

Since the reaction was performed in a dispersion system, the nanoparticle size could be obtained (Hayat *et al*., 2016). A dark yellow suspension indicated the formation of CdS particles. TEM images of the prepared CdSNPs (Figure 2) show that the nanoparticle size was around 7 nm.

The CdSNP was then conjugated with aflatoxin antibody, based on the procedure introduced by Valera et al. (2012). EDC and NHS were employed to provide the active sites for the CdS, so that the aflatoxin antibody could be attached to the CdSNPs. BSA was then added to cover the excess active sites.

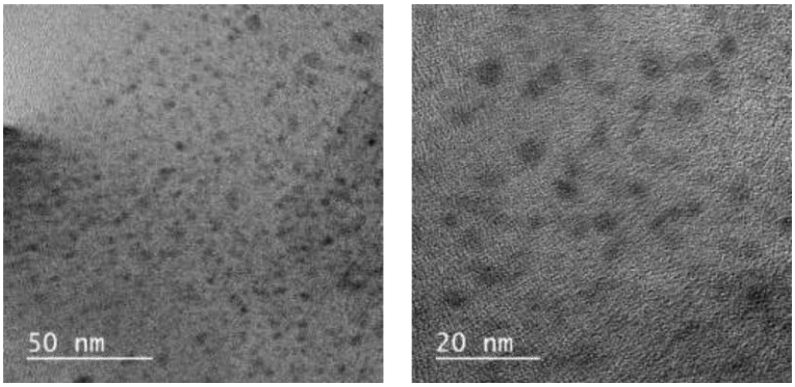


Figure 2 TEM images of the CdSNPs prepared by microemulsion

To characterize the conjugation between the CdSNPs and anti-aflatoxin, absorbance measurement was conducted by UV-Vis spectrophotometry in the wavelength range 350–600 nm (Figure 3). The CdSNPs show a sharp peak at around 470 nm due to the surface plasmon resonance of the nanoparticles. This peak shifted and broadened at around 500 nm, indicating the presence of a new bond between the nanoparticle and antibody (Sharma et al., 2010).

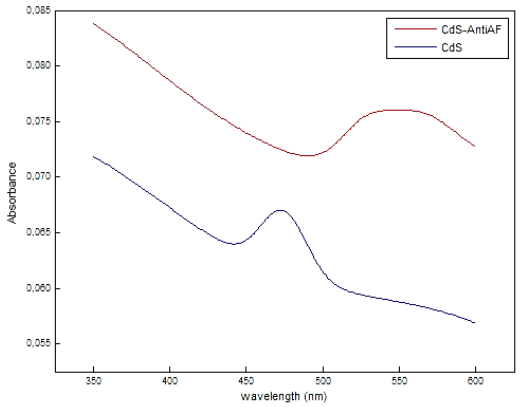


Figure 3 UV-Vis spectra of CdSNP and anti AFB-conjugated CdS NP

3.2. ASV Optimization and Measurement

ASV of Cd^{2+} ions in 0.1 M $HClO_4$ was performed to optimize the measurement parameters. In our previous report, the oxidation peak of Cd^{2+} in 0.1 M $HClO_4$ was obtained at −0.6 V (vs. Ag/AgCl). A deposition potential of −1.0 V (vs Ag/AgCl) with a deposition time of 60 s was applied, and the scan rate for the stripping step was 100 mV/s (Hayat et al., 2016). To optimize the parameters, in this work the deposition potential was varied in the range −1.0 to −3.0 V. Plots of the peak current against the Cd^{2+} concentration are summarized in Figure 4a.

It was clear that in the potential range between −1.0 and −2.5 V, the ASV slope increased with the deposition potential, while at a deposition potential of −3.0 V it decreased compared to that at a potential of −2.5 V. Furthermore, at a potential of −3.5 V and above, the response was undetected. Accordingly, a potential of −2.5 V was selected for subsequent measurements.

The deposition time was varied using an applied potential of −2.5 V in the time range of 60–600s. A summary of the peak currents versus the Cd^{2+} concentration is presented in Figure 4(b). The slope increased with the deposition time. However, at a deposition time of 360 s, the peak current decreased at a high Cd^{2+} concentration. Moreover, the deposition time could not exceed 360 s due to the device limitations. Therefore, the time for subsequent measurements was fixed at 300s.

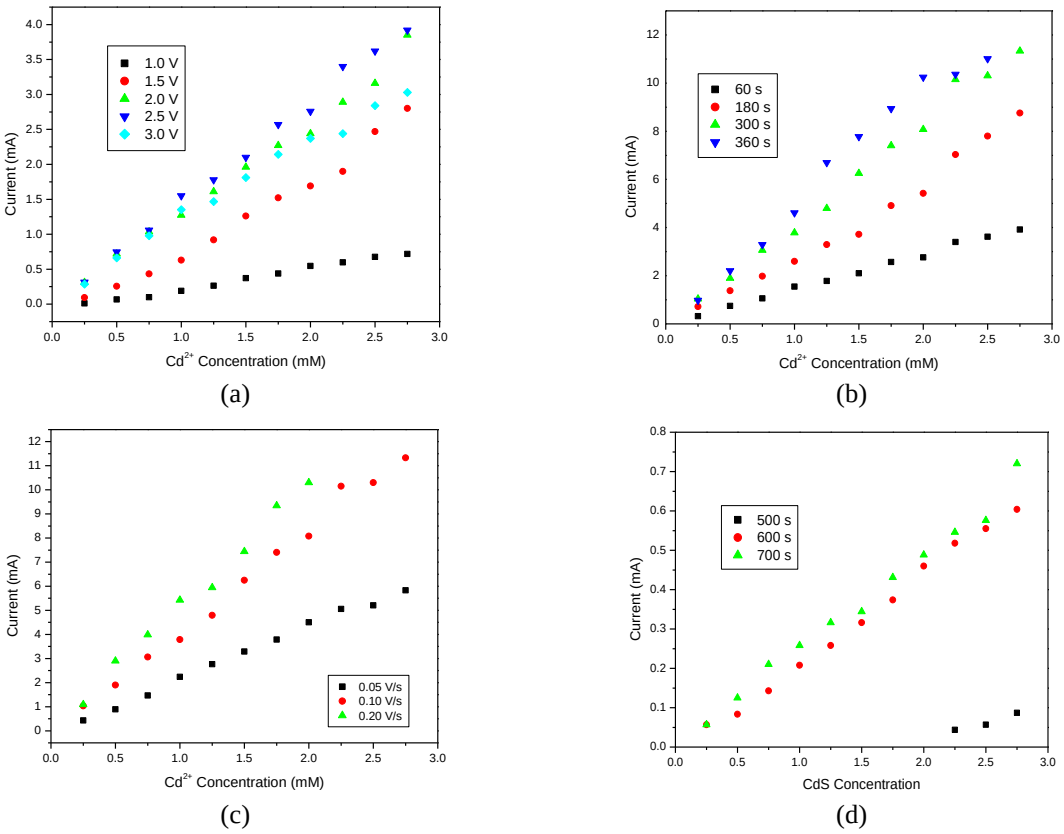


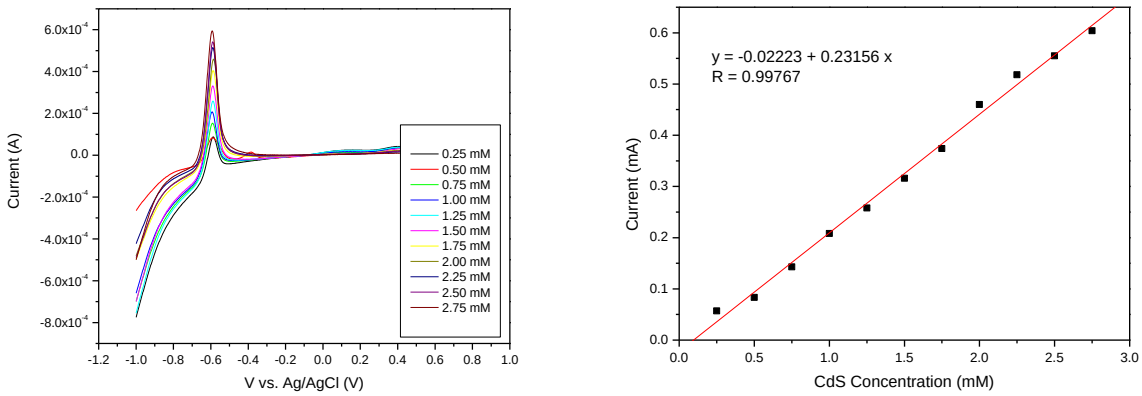
Figure 4 Anodic Stripping Voltammogram of Cd^{2+} in: (a) various deposition potentials; (b) deposition times; (c) scanrates; and (d) re-optimizing the measurement of CdS nanoparticles at deposition time variations of 500, 600, and 700 s

The scan rate was optimized in the range of 0.05–0.20 V/s. A summary of the peak current vs. the Cd^{2+}concentration is presented in Figure 4c. This clearly shows that the ASV slope increased with the scan rate. At a scan rate of 0.2 V/s, an ASV response was detected with a Cd^{2+} ion concentration of up to 2.0 mM. However, at higher concentrations, no response was detected due to the device limitations. Based on these results, the scan rate for the following experiment was fixed at 0.1 V/s.

However, when ASV measurements of the CdSNPs were conducted in the concentration range of 0.625–3.750 mM in 0.1 M $HClO_4$ under optimum conditions (scan rate 0.10 V/s, deposition time 300s and deposition potential −2.5 V), the ASV measurement produced no peak current for the CdSNPs. To resolve this problem, the deposition time was re-optimized by increasing it to 700s. The result is displayed in Figure 4d; based on this, the deposition time used for further experiments was 600s.

CdSNPs measured as Cd^{2+}ions were reflected by peak formation at −0.6V, corresponding to the peak of Cd^{2+} shown in Figure 5a. This was consistent with our previous result (Hayat et al., 2016). In $HClO_4$ solution, CdSNPs decomposed into Cd^{2+} and S^{2-} ions, according to the following reaction:

$$CdS + 2\ HCl0_4 \rightarrow Cd^{2+} + H_2S + 2\ Cl0_4^- \quad (2)$$



(a) (b)

Figure 5 Anodic stripping voltammograms as responses of concentration variations of CdS(a) and the related calibration curve (b). Measurements were performed in 0.1 M $HClO_4$ solution with a scan rate of 0.10 V/s, deposition time of 600 s, and deposition potential of -2.5 V

Plots of the oxidation peaks at −0.6 V against the CdS concentration are shown in Figure 5b; they show high linearity, indicating that CdS could be determined electrochemically using this method. In this case, CdSNPs can potentially be applied as labels in aflatoxin test strips. For this purpose, they were conjugated with anti-aflatoxin through an antigen–antibody reaction. CdSNP–anti-aflatoxin will be bound by the aflatoxin in the sample. The application of CdS as a label on the test strip provided both qualitative and quantitative measurements. Qualitatively, this was determined by the formation of a yellow colour in the test zone, while quantitatively it was determined electrochemically. The CdS concentration measured by ASV reflects the aflatoxin concentration in the sample.

3.3. Aflatoxin Test Strip Fabrication and Investigation

The aflatoxin test strip was arranged on a nitrocellulose membrane equipped with a rigid thin plastic sheet on the back cover as a support and adhesive for the sample pad, release pad and absorbent pad. Each pad was arranged (sample pad, conjugate pad and absorbent pad) on the back cover. The conjugate pad used in this test strip was immobilized by the CdSNP–anti-aflatoxin conjugate, composed of three drops (each 50µL). To ensure that the CdSNP–anti-aflatoxin conjugate was immobilized on the conjugate pad, each component was characterized using UV-Vis spectrophotometry at a wavelength of 350–600 nm, as shown in Figure 6. Comparing Figure 6 with Figure 3 confirms that the CdSNP–anti-aflatoxin conjugate was successfully immobilized on the conjugate pad.

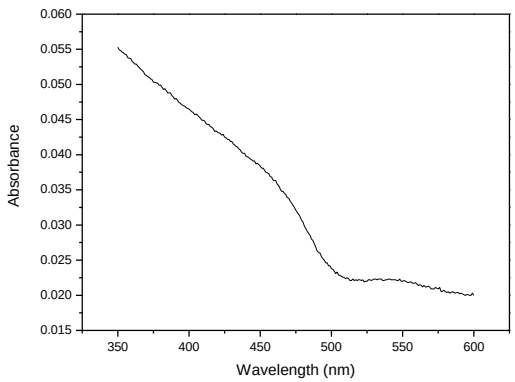
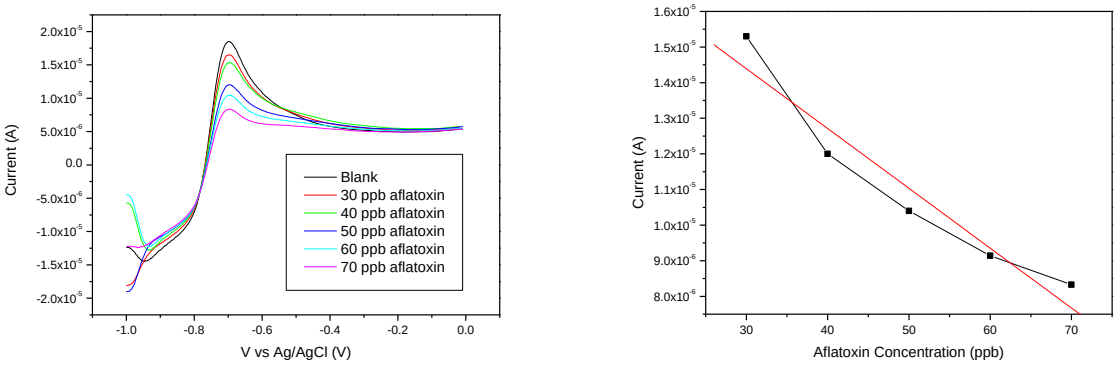


Figure 6 UV-visible spectrum of the conjugate CdS-NP – anti aflatoxin at a wavelength range of 350 to 600 nm

Furthermore, the prepared test strip was examined to determine the aflatoxin standards from concentrations of 0 (blank) to 70 ppb by ASV. A volume of 150 µL CdSNP–anti-aflatoxin conjugate was immobilized on the conjugate pad. The test was conducted by dropping the aflatoxin standard onto the sample pad and leaving it for 10 min to ensure that the sample moved from the sample pad towards the test zone and absorbent pad. From visual observations, none of the samples changed color in the test zone. However, examination by ASV using the test strip parameters in Figure 7a showed a peak at around −0.6 V, which was similar to those in the voltammograms in Figure 5, indicating that Cd^{2+}in the form of a CdSNP–anti-aflatoxin conjugate in the test strips was measured.



(a) (b)

Figure 7 (a) Stripping voltammograms of the strip test prepared with 150µL of CdSNP-antiaflatoxin in the conjugate pad and 0.4 ppb aflatoxin in the sample pad; and (b) the related linear curve of the aflatoxin standard sample in the concentration range of 0-70 ppb

Figure 7a also shows that the oxidation peak in the voltammograms decreased as the aflatoxin concentration increased. Plots of the peak currents against the aflatoxin concentration (Figure 7b) yield the linear equation $y = 1.94\times10^{-5} - 1.68\times10^{-7}x$ ($R^2 = -0.96$) with a detection limit of 30 ppb, indicating that the method is potentially suitable for determining aflatoxin in real-world applications.

4. CONCLUSION

ASV of Cd^{2+} in 0.1 M $HClO_4$ showed positive results when applied to CdSNP-based aflatoxin biosensors. A deposition potential of −2.5 V with a deposition time of 700 s and a scan rate of 100 mV/s generated an oxidation peak at −0.6 V. A linear correlation was observed between the decrease in the current peak and the increase in aflatoxin concentration in the range of 0–70 ppb, indicating that the method is promising for aflatoxin determination. The test strip was measured by anodic stripping voltammetry using a boron-doped diamond electrode. A linear concentration range of 0–70 ppb aflatoxin, with a sensitivity of 20 µA/mM and a detection limit of 30 ppb, was achieved by this method.

5. ACKNOWLEDGEMENT

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