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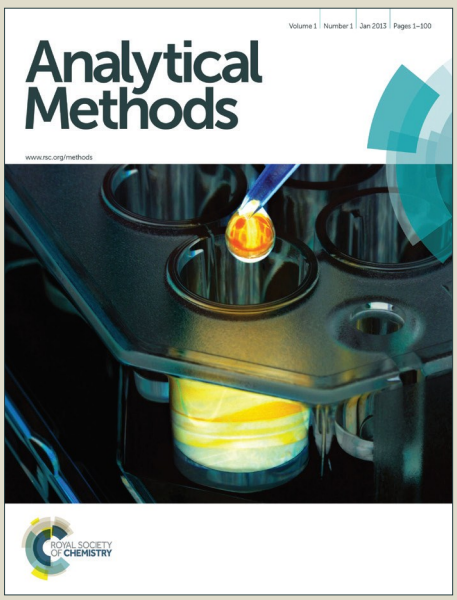

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Selective and Sensitive Determination of Ochratoxin A Based on Molecularly Imprinted Electrochemical Luminescence Sensor

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This paper reports a new molecularly imprinted electrochemical luminescence (MIP-ECL) sensor for the determination of ochratoxin A (OTA) with high selectivity and sensitivity. $Ru(bpy)_3^{2+}$ was immobilized on the electrode surface as the luminescent material by nafion. The process of template elution and rebinding acted as a gate to control the flux of probes, which passed through the cavities and reacted on the electrode surface. When the imprinted film was rebound with OTA, the ECL signal decreased. Under the optimal conditions, the MIP-ECL sensor showed wide linear range from 0.1 ng/mL to 10 ng/mL with a detection limit (S/N= 3) of 0.03 ng/mL. Corn samples were assayed by using this sensor, and the recoveries ranging from 95.2% to 102.7% were obtained.

1. Introduction

Ochratoxin A (OTA), a secondary fungal metabolite, is generated primarily by several Aspergillus and Penicillium genera.[1] It is hepatotoxic, nephrotoxic, teratogenic and immunotoxic to several animal species and a possible carcinogen to humans.[2] It also can contaminate a variety of food commodities, including corns, fresh grapes, dried vine fruits, wine, beer, coffee, spices, cocoa et. al.[3] To reduce its risks of human and animal health, monitoring the presence of OTA in food and feed is advisable and necessary. Currently, the most widely applied methods for OTA detection rely on thin-layer chromatography (TLC)[4], capillary electrophoresis (CE)[5], chemiluminescence (CL)[6], fluorescence (FL)[7], high performance liquid chromatography (HPLC)[8] and enzyme-linked immunosorbent assay (ELISA)[9]. Among them, TLC and CE methods are poorly sensitive, while FL methods could obtain higher sensitivity than that of TCL, but they lack selectivity. HPLC methods are sensitive and selective, but they require expensive apparatus and complex procedures for sample pretreatment. And for ELISA, although a high sensitivity can be achieved, the poor chemical/physical stability of the antibodies or enzymes prevents their use in the harsh environments of acids or bases and organic solvents.

Recently, Electrochemical luminescence (ECL) has played an essential role in the fields of environment[10], immunoassays[11] and pharmaceutical analysis[12] owing to its high sensitivity, low background, and no need for expensive instruments. $Ru(bpy)_3^{2+}$ as a conventional ECL reagent has been extensively used in cancer detection[13] and pharmaceutical analysis[14]. It also has great potential for developing novel ECL sensors.[15] To reduce the consumption of expensive ECL reagent $Ru(bpy)_3^{2+}$, it takes high priority in use of anchoring $Ru(bpy)_3^{2+}$ on the electrode over the solution. Nafion has excellent chemical stability, mechanical strength, ion transport function and selectivity for some hydrophobic cation. And $Ru(bpy)_3^{2+}$ is easy to access the hydrophobic region of nafion in order to be immobilized. But nafion film is dense, which contributes to the slow mass transfer rate. So, multi wall carbon nanotubes (MWCNTs) are chosen to be doped into nafion to improve the redox current and ECL signal. The MWCNTs/Nafion film has a more open structure and a larger surface area, which has a beneficial effect on the diffusion of $Ru(bpy)_3^{2+}$ in the film.

Molecular imprinting is a unique technique used for preparing polymers with synthetic recognition sites that have a predetermined selectivity for analytes of interest.[16] The molecularly imprinted polymers (MIP) have been utilized as materials of molecular recognition in many scientific and technical fields, such as solid-phase extraction, chromatographic separation, membrane separations, sensors, drug releases, and catalysts.[17-22] Considering its merits of predetermine[23], identification[24] and practicality[25], molecular imprinting is combined with ECL to produce a sensor with both high sensitivity and desirable selectivity, which can help to solve the problem related to the selectivity of ECL.[26]

In this work, a novel MIP-ECL sensor for the determination of OTA with high selectivity and sensitivity was composed (scheme 1). The MWCNTs/Nafion was used to immobilize $Ru(bpy)_3^{2+}$ on the electrode. The cavities that were produced after elution in the film acted as the channels for electron transportation. When the imprinted film rebound with OTA, the ECL signal decreased. The

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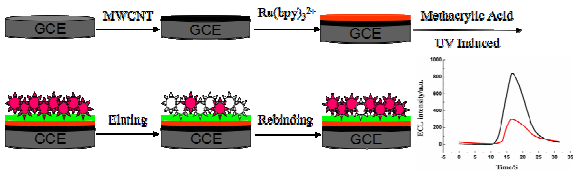
prepared sensor was successfully applied to the detection of OTA in corn samples.



Scheme 1. The fabrication process of the MIP-ECL sensor and the detecting of OTA.

2. Experimental

2.1 Chemicals and reagents

All reagents were used of analytical grade and double distilled water was used to prepare sample solutions. Ochratoxin A was purchased from cayman chemical company (United States). Tripropyl amine (TPrA) and nafion were obtained from Sigma-Aldrich (Beijing, China). MWCNTs and Terpyridyl ruthenium were obtained from Cheng du Organic Chemicals Co. Ltd. (Chengdu, China) and Green kaemmer (Beijing, China), respectively. The supporting electrolyte was 0.1 mol/L phosphate buffer solution (PBS) prepared with Na_2HPO_4 and KH_2PO_4 and the pH was adjusted with NaOH or H_3PO_4. Corn samples (8.6 μg/kg ± 3.6 μg/kg, HPLC) were purchased from Biopure (New Zealand).

2.2 Apparatus

ECL signals were measured with MPI-E electrochemiluminescence analyzer (Xi'An Remax Electronic Science &Technology Co. Ltd., China). Scanning electron microscopy (SEM) was performed using a JEOL JSM7100F SEM facility. Fluorescence was measured with LS-55 fluorescent spectrophotometer (USA). A three-electrode system was used in all measurements, with a glassy carbon electrode (GCE, 3 mm in diameter) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a platinum wire as the auxiliary electrode. Branson 2000 ultrasonic cleaner (USA) was used to clean the electrodes.

2.3 Preparation of MIP-ECL sensor

Before modification, the GCE was polished with 0.3 and 0.05 mm alumina slurry and rinsed thoroughly with double distilled water between each polishing step. Then, it was washed successively with double distilled water and ethanol in an ultrasonic bath, and finally dried at room temperature.

Firstly, 1 mg of MWCNTs were added into 1 mL of 0.5 wt% nafion solution and ultrasonically dispersed for 20 min to obtain a homogeneous, well-distributed solution of the MWCNTs/nafion complex. The GC electrode was coated with 2 μL MWCNTs/Nafion suspension (1 mg/mL), and the solvent was allowed to evaporate at room temperature. Next the modified GC electrode (MWCNTs/Nafion/GCE) was coated with 5 μL 10 mM $Ru(bpy)_3^{2+}$ aqueous solution and placed in room temperature for evaporating. $Ru(bpy)_3^{2+}$/MWCNTs/Nafion/GCE was obtained.

Secondly, template molecule of OTA was mixed with the functional monomer methacrylic acid (MAA), the crosslinking agent of ethylene glycol dimethacrylate (EDMA) and initiator azodiisobutyronitrile (AIBN) to obtain the initial solution. Then 2 μL of the initial solution was dropped on the $Ru(bpy)_3^{2+}$/MWCNT/nafion/GCE and the polymer reaction was induced by the wavelength of 245 nm ultraviolet light. The MIP-ECL sensor was obtained by ethanol elution of the imprinted polymer film. In addition to the template molecule, other preparation process of the non imprinted electrochemical luminescence (NIP-ECL) sensor was in accordance with the MIP-ECL sensor.

2.4 Elution, Incubation, and ECL measurement

After polymerization, the MIP-ECL and NIP-ECL sensors were washed with ethanol for 3 min to remove the imprinting molecules or the adsorbates within or on the surface of the imprinted film, and then the MIP-ECL sensor with stereo cavities in the imprinted was obtained. Next the MIP-ECL sensor was immersed in 2 mL of 0.1 ng/mL to 10 ng/mL OTA for 10 min to rebind all the vacant binding cavities in MIP. Finally, the ECL measurements were conducted in a 2 mL 0.1 M PBS (pH 7.0) containing 0.1 mM tripropyl amine (TPrA)[15,27-29]. The ECL test was performed by CV from 0 to +1.6 V with the scan rate of 100 mV/s. The voltage of the photomultiplier tube (PMT) was set at 600 V.

3. Results and discussion

3.1 Characterization of MIP-ECL sensor

Scanning electron microscope (SEM) was performed to characterize the morphologies of the different modified electrodes, and the results are shown in Figure 1. Figure 1a was the bare GCE. Then MWCNTs were attached with nafion solution to the surface of GCE, and the tubular structure of MWCNTs appeared (figure 1b). when $Ru(bpy)_3^{2+}$ was coated on the surface of MWCNTs/Nafion/GCE, the layered structure of $Ru(bpy)_3^{2+}$ crystal can be seen (figure 1c). It can be observed from figure 1d that a dense film was covered onto the surface of the $Ru(bpy)_3^{2+}$/MWCNTs/Nafion/GCE after UV polymerization process.

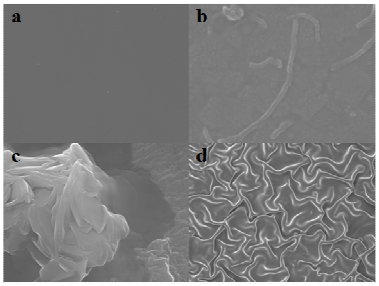


Figure 1. SEM images of the modified electrode. (a) The bare electrode, (b) MWCNTs/Nafion/GCE, (c) $Ru(bpy)_3^{2+}$/MWCNTs/Nafion/GCE, (d) MIP/$Ru(bpy)_3^{2+}$/MWCNTs/Nafion/GCE.

Electrode impedance spectroscopy (EIS) is an effective and convenient technique for probing the feature of the modified electrode surface. Here, EIS was used to investigate the change of resistance after each constructional step, as shown in figure 2A. Because of the positively charged $Ru(bpy)_3^{2+}$, the charge transfer resistance for $Ru(bpy)_3^{2+}$/MWCNT/Nafion/GCE (curve b) showed a decrease compared with that of MWCNT/Nafion/GCE (curve a). The $Ru(bpy)_3^{2+}$/MWCNT/Nafion/GCE was coated with the imprinted polymer, which made the electron transfer difficult between the

bulk solution and the electrode surface, so the resistance increased obviously (inset, curve c). After removal of the template molecule, the resistance decreased. This is more likely a consequence of the fact that the formed cavities on the MIP could facilitate the electron transfer. when the OTA rebound with imprinted film by immersing the MIP-ECL sensor in the standard solution of 5 ng/mL OTA for 10 min, the channels were blocked, and the resistance increased (curve e).

Fluorescence spectrum (FLS) was used to attest the process of elution and rebinding of the imprinted polymer (figure 2B) owing to the fluorescence of OTA. The imprinted film was obtained with a high concentration of OTA, and its intensity was highest (curve a). When the imprinted film was washed with ethanol for 3 min to remove the template molecule of OTA, the fluorescence intensity decreased (curve b). Then the imprinted film was immersed in 5 ng/mL OTA for 10min, OTA could be recombined on the imprinted film, and the fluorescence intensity increased (curve c).

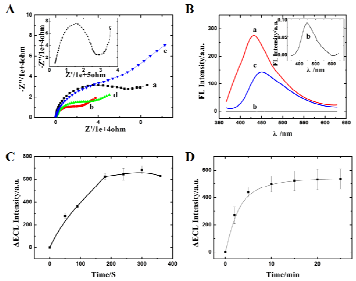


Figure 2. (A) Electrochemical impedance plots of different electrodes. (a) MWCNT/Nafion/GCE, (b) $Ru(bpy)_3^{2+}$/MWCNT/Nafion /GCE, (c) MIP/$Ru(bpy)_3^{2+}$/MWCNT/Nafion/GCE before template removal, (d) MIP/$Ru(bpy)_3^{2+}$/MWCNT/Nafion/GCE after template removal, (e) MIP/$Ru(bpy)_3^{2+}$/MWCNT/ Nafion/GCE after rebinding. (B) Fluorescence spectrum of the process of elution and rebinding of the imprinted polymer. (a) The imprinted polymer before elution. (b) The imprinted polymer after elution. (c) The imprinted polymer after rebinding. Inset: the enlargement fluorescence spectrum of imprinted polymer after rebinding. Effects of (C) masking time and (D) rebinding time on response signals.

3.2 ECL behavior of MIP-ECL sensor

Since cavities in MIP can be used as the channels for electron transportation, the blocking of channels causes the change in the ECL intensity, correspondingly. As shown in figure 3, when a nonconductive MIP was formed on the GCE electrode surface, it was difficult for $Ru(bpy)_3^{2+}$ to touch with TPrA, and the ECL intensity was small (curve a, written I_0). After the removal of the template molecule, some cavities in MIP appeared and the ECL intensity increased (curve b, written I_1). When the MIP electrode continuously rebounded with OTA, the cavities were blocked again and the ECL intensity decreased (curve c, written I_2). But it was much higher than that of curve a, because some cavities might be deformed, which made it difficult for the template molecules to enter the cavities. The insert in figure 3 was the ECL of NIP-ECL sensor. When the non imprinted polymer was formed on the surface of the electrode, the ECL intensity was almost zero. It is because that the non imprinted polymer was nonconductive for the electrochemical reaction (curve a'). After the elution step, the ECL intensity of curve b' changed little compared with that of curve a'. It maybe has some small molecules that also can be eluted by ethanol on the non imprinted polymer. It demonstrated that few cavities in the non imprinted polymer can be formed as the channels for reaction of $Ru(bpy)_3^{2+}$ and TPrA after elution.

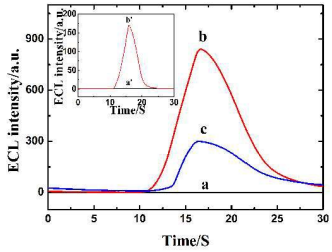


Figure 3. ECL curves of (a)MIP/$Ru(bpy)_3^{2+}$/MWCNTs/Nafion/GCE before template removal, (b) MIP/$Ru(bpy)_3^{2+}$/MWCNTs/Nafion/GCE after template removal, (c) MIP/$Ru(bpy)_3^{2+}$/MWCNTs/Nafion/GCE after rebinding. Inset: the ECL curves of (a') NIP/$Ru(bpy)_3^{2+}$/MWCN-Ts/Nafion/GCE, (b') NIP/$Ru(bpy)_3^{2+}$/MWCNTs/Nafion/GCE after template removal.

3.3 Optimization of experimental conditions

The eluting process was implemented in 2 mL of ethanol. The results showed that the ECL intensity increased as the elution time increased from 0 to 3 min (Figure 2C), after that the responses (I_1-I_0) reached a plateau. Thus, the elution time chosen for removing the template molecule was 3 min.

The rebinding process was carried out in 2 mL of 5 ng/mL OTA. ECL was preformed every 5 min in the experiment. The ΔI_{ECL} intensity ($\Delta I_{ECL} = I_1 - I_2$) reached the maximum when the rebinding time reached 10 min (Figure 2D). As a result, 10 min was selected as the rebinding time in all of the following assays.

3.4 ECL response to OTA

Under the optimized conditions, the MIP-ECL sensor was immersed in the OTA solution of different concentrations, and the ECL intensity of before rebinding and after rebinding were recorded. With the increase of the concentrations of OTA for rebinding, the ΔI_{ECL} of the $Ru(bpy)_3^{2+}$ system obviously increased. And the ΔI_{ECL} was linear to the concentrations (C) of OTA in the range from 0.1 to 10 ng/mL (Figure 4A) with a detection limit of 0.03 ng/mL (S/N=3). The linear regression equation was ΔI_{ECL} = 15.76 C (ng/mL) + 488.68, with a coefficient of correlation, R = 0.996.

3.5 Reproducibility, Selectivity and Stability

In order to evaluate the selectivity of the MIP-ECL sensor, some mixed solutions were prepared. We prepared the MIP-ECL sensor by the same method, but then the electrodes were incubated in different mixed solutions, which were 5 ng/mL OTA mixed with 100 ng/mL ochratoxin B (OTB), 100 ng/mL deoxynivalenol (DON), and 10 μM glucose (GLU), respectively. OTA, OTB and DON belong to the same class of mycotoxin and moreover OTB has similar structure as OTA. GLU is a kind of interfering substance that exists in corn samples. At last, we detected the electrodes that had been treated with the mixed solutions in the same solutions. The results showed that there were no evident

difference between the mixed solutions and OTA (Figure 4B). They also reflected that the MIP-ECL sensor had excellent selectivity.

To evaluate the reproducibility of the MIP-ECL sensor, we prepared ten electrodes under the same conditions before rebinding with OTA, independently. All detecting of the ten electrodes were performed in 0.1 M PBS PH 7.0 containing 0.1 mM TPrA. As shown in figure 4C, we found that the ECL intensities of the ten electrodes were almost the same (RSD=4.43%). This proves that the MIP-ECL sensor was stable and reproducible.

To investigate the stability of the MIP-ECL sensor, the needed sensors were prepared in the same way and then stored in the fridge. Subsequently, the electrodes that had been immersed in 5 ng/mL OTA for 10 min were detected in the same solutions day by day. The results showed that they had no significant difference between everyday (RSD=5.29%) (Figure 4D), which revealed good stability.

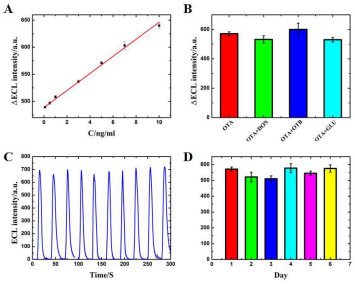


Figure 4. (A) Calibration curves for OTA determination based on ΔI_{ECL} as the response signal. The inset showed the ΔI_{ECL} depended linearly on the concentration of OTA in the range from 0.1 ng/mL to 10 ng/mL under the optimized conditions. The error bars represent the standard deviation of three independent measurements. (B) Selectivity of the MIP-ECL sensor to OTA, OTB, DON, GLU. (C) ECL curves of ten different electrodes. (D) The stability of the MIP-ECL sensor.

3.6 Determination of OTA in corn samples

Table 1. Detection of OTA in corn samples by the MIP-ECL sensor.

Corn samples (ng/mL)	OTA added to the corn samples (ng/mL)	OTA detected (ng/mL)	Recovery (%)	RSD (*n*=3) (%)
2.58 (RSD=5.7%)	3	5.52	98.0	5.7
	5	7.34	95.2	3.5
	7	9.75	102.4	8.8

The corns (0.5 g) that contain OTA were immersed in the solution of 2 mL 75% (V/V) methanol/water, and then ultrasonically dispersed for 2 h. After centrifuged at 12000 rpm for 10 min, the resulting suspension was the corn sample including OTA. The average measured concentration of OTA was 10.32 μg/kg by the MIP-ECL sensor in this work, which was in agreement with the result of HPLC. In addition, the standard addition method was used to test the reliability of the MIP-ECL sensor for determination of OTA in corn. The results showed that the recoveries of OTA were between 95.2% and 102.7%, and the RSD ranged from 3.5% to 8.8% (Table 1).

4. Conclusions

In this work, we demonstrated a MIP-ECL sensor for ultrasensitive detection of OTA. The MIP-ECL sensor combined molecularly imprinted technology with electrochemiluminescence could improve not only the sensitivity of electrochemiluminescence, but also the selectivity of molecularly imprinted technology. Under the optimal conditions, this sensor could detect OTA in the range from 0.1 ng/mL to 10 ng/mL with a detection limit of 0.03 ng/mL (S/N= 3). Moreover, this sensor also could detect OTA in corns with good accuracy, which makes us fully believed that it has great potential for practical applications.

Acknowledgements

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