

# The Assembly of a Novel Enzyme Biosensor for Aflatoxin B<sub>1</sub> Detection\*

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**Abstract** A novel biosensor for aflatoxin B<sub>1</sub> detecting has been reported. The biosensor electrode for AFB<sub>1</sub> detecting was assembled by immobilized aflatoxin-oxidoreductase using open-ended multi-walled carbon nanotubes as matrix. Its linear range was between 0.16 μM and 3.2 μM. And if the specific anti-aflatoxin B<sub>1</sub> antibody and aflatoxin oxidoreductase were both immobilized on the electrode with Multi-Walled carbon nanotubes, the detection limit of the modified electrode could be 16 nM with a 10 times improved sensitivity. The aflatoxin enzyme biosensor assembled this way strode one step forward its practical application.

**Key words** Aflatoxin B<sub>1</sub> Enzyme electrode Multi-walled carbon nanotubes

Aflatoxins, secondary metabolites from *Aspergillus flavus* and *A. parasiticus*, were a group of carcinogens commonly found in food and feed stuffs. Because of their mutagenic, aflatoxin toxinogenic bacteria could contaminate a wide-range of agricultural products and be stable under extremely high temperatures. Therefore the fast and convenient detection of aflatoxin were urgently needed.

Due to their rapidity, high sensitivity and convenience, assembling biosensors were the alternative methods for fast-detection. Many bio-materials including liver tissue, photorhabdus luminescens reporter fused whole cells of *Escherichia coli* and the anti-aflatoxin antibody had been used to detect aflatoxins. Validation of the procedure for the determination of aflatoxin B<sub>1</sub> in animal liver using immunoaffinity columns and liquid chromatography with postcolumn derivatisation and fluorescence detection was studied. The limit of detection was 2 ng/kg, and the linear range was between 0.002 μg/kg to 1 μg/kg<sup>[1]</sup>.

Up until now, anti-aflatoxin antibody biosensor had been the most sensitive detecting method because of its higher selectivity and sensitivity.

However, the difficulties of regeneration and reutilization of biosensor couldn't be solved easily and thoroughly<sup>[2,3]</sup>, and so these immunosensors were designed mostly disposable and batch-analytic<sup>[4,5]</sup>. In addition, the cost and preparation of some biosensors were high and complicated, respectively.

In contrast with the immune biosensors, enzyme biosensors did not have the regeneration problems since the substrate could spontaneously dissociate after enzyme catalysis, and they were simple, portable and low-cost mostly. This spontaneous dissociation property also made enzyme biosensors suitable for continuous-flow-analysis. In addition, the enzyme biosensor could also be used for multi-substance detection because of the characteristic redox peak value ranges of various substrates. However, the sensitivity of the enzyme sensors was often lower than the immunosensors for the lower affinity between the enzyme and its substrate. Assembling high sensitivity biosensor in different ways was always an important content of enzyme biosensor study.

Previously, we had isolated an enzyme from *Armillariella tabescence*, which was confirmed to have a detoxifying activity towards aflatoxin B<sub>1</sub> (the enzyme has

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been previously named aflatoxin detoxifzyme, ADTZ)<sup>[6-8]</sup>. In further study it had been found to be an oxidoreductase (in this study it was called aflatoxin oxidoreductase). This ADTZ had been used to construct an enzymatic multi-walled carbon nanotubes (MWNTs)-Au electrode to detect Sterigmatocystin (ST) (a precursor of AFB<sub>1</sub>, with the same bis-furan ring structure) in our previous investigations<sup>[9,10]</sup>. The electric signal produced via ADTZ Sterigmatocystin catalyzation indicated that the detection limitation of biosensor could reach to 10<sup>-5</sup> (g/L). In this study, open-ended multi-walled carbon nanotubes were used as the immobilizing matrix to stabilize ADTZ, and then assembled on Au electrode to detect AFB<sub>1</sub>. And attempts of assembling Au electrode by immobilized ADTZ, specific anti-AFB<sub>1</sub> antibody and MWNT had been done to improve the sensitivity of the aflatoxin detoxifzyme biosensor.

## 1 Materials and Methods

### 1.1 Materials

Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) was purchased from Fermentek Co. (Israel), Anti-aflatoxin B<sub>1</sub> anti-body [anti-aft Antibody (Ab)] was from Sigma Co. (USA). Sterigmatocystin (ST), N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimi dehydrochloride (EDC) and N-Hydroxysuccinimide (NHS) were obtained from Sigma-Aldrich; Multi-walled carbon nanotubes (MWNTs) were from Nano Port Co. (China) and other pure analytical reagents were domestically produced.

### 1.2 Preparation of Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) oxidoreductase for electrode modification

The preparation of AFB<sub>1</sub> oxidoreductase followed similar procedures described previously<sup>[7]</sup>. After the final concentration step, AFB<sub>1</sub> oxidoreductase was 0.593 g protein/L with an activity of 2.40 × 10<sup>-4</sup> U (1U was equal to the amount of enzyme that can decrease 1 μmol of AFB<sub>1</sub> per minute).

### 1.3 Preparation of enzyme-MWNTs linked electrode

The MWNTs base electrode was made as previous<sup>[9,10]</sup>. After oxidative activation, MWNTs were suspended in N, N-dimethylformamide (DMF) at 0.1mg/

ml followed by ultrasonic homogenization. The gold electrode polished with toothpaste and diatomite was subsequently ultrasonically cleaned in acetone, sulfuric acid (0.5mol/L) and concentrated nitric acid (1:1) for 3 ~ 5 min each time. Nanotube films were prepared by dropping 10 μl of the MWNTs suspension onto the polished gold electrode and then evaporating the solvent in oven at 60°C<sup>[11]</sup>. The successful of MWNTs modified on Au electrode has been confirmed by detecting Phosphate buffer solution (PBS) (0.1mol/L pH6.5) solution.

### 1.4 Covalent immobilization of enzyme (with or without Ab) on the MWNTs-modified electrode

Two types of electrodes, enzyme-MWNTs and enzyme-Ab-MWNTs have been made. The MWNTs base electrodes were immersed sequentially in 100g/L EDC and 100g/L NHS solution (both EDC and NHS were dissolved in 0.01mol/L PBS pH 7.4) for 30min respectively. After EDC-NHS treatment, the base electrode surface was immersed in the aflatoxin-oxidoreductase solution at a concentration of 0.593 g protein/L (containing 2 μl Polyethylene glycol (PEG)-200 and 10 ppb of AFB<sub>1</sub>), then the electrode was laid on its side for 30 min. to dry at ambient temperature. The biosensor electrode of enzyme-MWNTs-Au was thus formed by methods reported previously<sup>[12]</sup>. Before using, the electrode should be washed in 0.01mol/L PBS pH 7.4 three times.

The enzyme-Ab-MWNTs electrode was constructed by immersing the EDC-NHS treated MWNTs base electrode into an Ab-enzyme mixture solution. The enzyme was at the same concentration used for the enzyme-MWNTs electrode and the anti-AFB<sub>1</sub> Ab used was 0.085mg/ml in the proportion of 1:5 (v:v) enzyme:Ab, (containing 2 μl PEG-200 and 10 μg/L of AFB<sub>1</sub>). The surface morphology of MWNTs base and enzyme-Ab-MWNTs modified electrodes were explored by Atomic Force Microscopy (AFM) (Autoprobe CP Research, Thermo Co., USA).

### 1.5 The electrochemical response of the enzyme-MWNTs-Au electrode to AFB<sub>1</sub>

The bio-analytical system BAS100 (BAS Co., USA) was used for amperometric measurements. The enzyme-MWNTs-Au electrode (the enzyme electrode) and the enzyme-Ab-MWNTs-Au electrode (the Enzyme-Ab-MWNT

electrode) described above, Ag/AgCl and a platinum wire were used as the working electrode, reference electrode and counter electrode, respectively. The electrochemical characterization of AFB<sub>1</sub> was studied in a methanol-PBS (55:45, v: v) solution by using cyclic voltammetry (CV) and differential pulse voltammetry (DPV) respectively. The electrode potential on the abscissa E/V was given versus the reference potential. The potential range was from -1000 E/mV to +1000 E/mV. All measurements were performed at 25°C.

### 1.6 The reusability study of the Enzyme-Ab-MWNT biosensor

The reusability of the constructed Enzyme-Ab-MWNT electrode was investigated by using the enzyme-Ab-MWNTs electrode to measure the response current of a 0.064  $\mu$ M AFB<sub>1</sub> methanol-PBS (55:45, v: v) solution repeatedly. To detect the remaining antigen (AFB<sub>1</sub>) on the electrode surface, the sample measurement was alternated with measurements of the response current of solvent control.

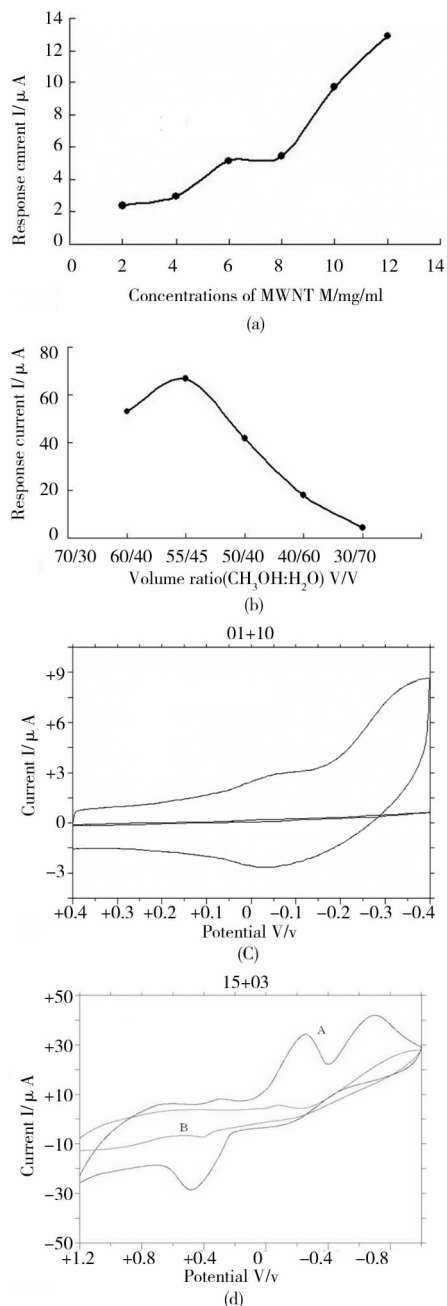
## 2 Results

### 2.1 Electrical behavior of the enzyme-MWNTs electrode

**2.1.1** The cyclic voltammetry diagram of the enzyme-MWNTs electrode in AFB<sub>1</sub> solution A study was carried out to determine the optimal concentration of MWNTs in electrode assembly. Several concentrations of the MWNTs were used in the immersing mixture and the electrode potentials measured with potassium ferricyanide (5mM) as the analyte in this step. The results indicated that 0.50mg/ml MWNTs was optimal since the electrode surface modified with higher concentrations of MWNTs was found to be unstable and flaked away easier [Fig. 1 (a)]. The conduction between MWNTs and the underlying gold surface can be shown as Fig. 1(c). This results confirmed the successfully of MWNTs modified on the Au electrode.

The first step in assay development was the selection of the buffer (PBS, methanol-PBS at various ratios (v: v): 70/30, 60/40, 55/45, 50/50, 40/60, 30/70). The best assay conditions were achieved with methanol:water (0.01mol/L PBS pH 6.5) 55:45 (v: v) [Fig. 1 (b)]. And, without the enzyme assembly no signals can be found

when using naked and MWNT modified Au electrode to detect AFB<sub>1</sub> solution (results not shown).



**Fig. 1 MWNTs-electrode assembly and assay conditions**

(a) Shows the effect of various concentrations of MWNTs (multi walled carbon nanotubes) in the assembly solution on the electrode response signal. (b) shows the assay conditions study; methanol/water at 55:45 (v: v) produced the greatest response sensitivity. The CV diagram of the naked and MWNTs modified Au electrode on PBS solution has shown as (c). And (d) shows the CV diagram of this enzyme-MWNTs electrode on A: 1.28  $\mu$ M AFB<sub>1</sub> (aflatoxin B<sub>1</sub>) solution and B: Methanol:water 55:45 (v: v) control

The cyclic voltammetry of blank methanol-PBS (v:v 55:45) and 1.28μM AFB<sub>1</sub> methanol-PBS (v:v 55:45) solution were measured by using the enzyme-MWNTs electrode [Fig. 1 (d)]. A significant reduction peak of AFB<sub>1</sub> appears at -650 E/mV as well as a pair of red-ox peaks at +400 E/mV and -250E/mV. These three peaks were about equal in strength. A pair of very weak redox peaks can also be found in the blank solution, however, they do not interfere with the sample determination since the reduction peak at -650 E/mV was chosen for the quantitative assay.

When 1.28μM AFB<sub>1</sub> (methanol-water) solution was scanned at various rates from 50 mV/s to 400 E/mV/s (results not shown), there was a linear relationship between the response currents and the square root of scanning rates, suggesting that the transfer of the substrate to enzyme film surface was mostly affected by linear diffusion, while the effect of sacrificial absorption on the electrodes surface was minor. Since faster scan rates led to a wider peak, 100 E/mV/s was chosen in the subsequent experiments.

2.1.2 The dependence of enzyme-MWNTs electrode response current on AFB<sub>1</sub> concentration The differential pulse voltammetry (DPV) character of the enzyme-MWNTs electrode for various AFB<sub>1</sub> concentrations from 0.16μM to 3.2μM is shown in Fig. 2 (a) and 2 (b). When using the DPV method to detect the response current, a rough linear relationship shown to AFB<sub>1</sub> concentration (C) within that range.

$$I/\mu A = 5.967C/\mu M + 1.241 \text{ (} R^2 = 0.9593, P < 0.05 \text{)}, \text{ (DPV data under } -650 \text{ E/mV)}$$

$$I/\mu A = 6.927C/\mu M + 1.479 \text{ (} R^2 = 0.9485, P < 0.05 \text{)}, \text{ (DPV data under } -250 \text{ E/mV)}$$

However, the determined peaks data under -650 E/mV shown a better linear relationship. The detection limit of the enzyme-MWNTs lies at 0.16μM AFB<sub>1</sub>. And the working range is form 0.16μM to 3.2μM.

2.2 The electrical behavior of the Enzyme-Ab-MWNT electrode

2.2.1 Enzyme-Ab-MWNTs electrode behavior on AFB<sub>1</sub> solutions by cyclic voltammetry To improve the enzymatic electrode sensitivity, an Enzyme-Ab-MWNT electrode has

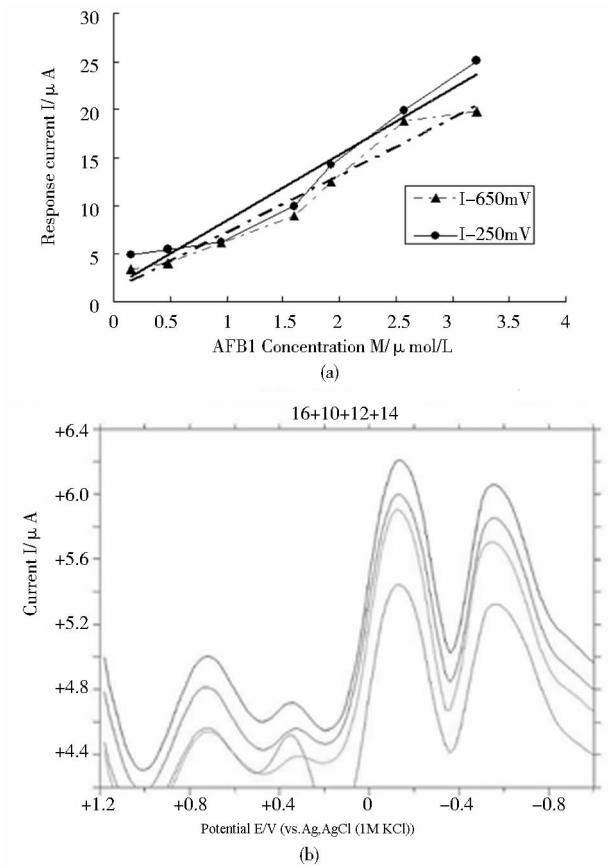
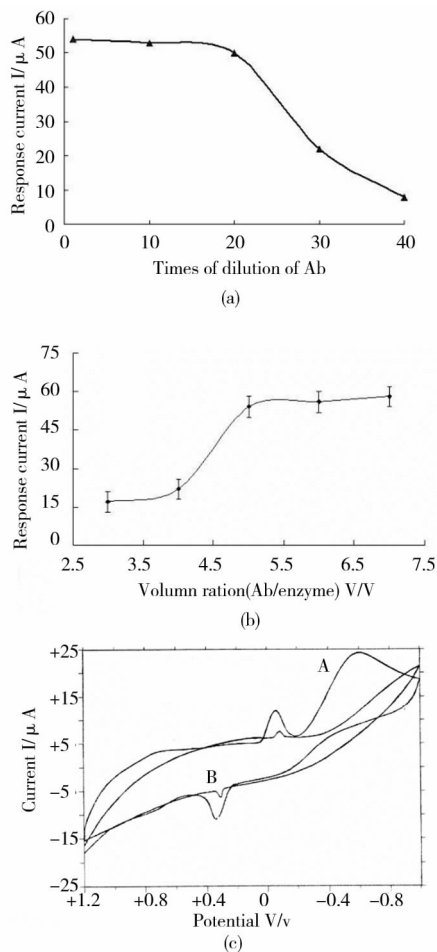


Fig. 2 The Enzyme-MWNTs electrode response currents

(a) Enzyme-MWNTs (multi walled carbon nanotubes) electrode response currents for various concentrations of AFB<sub>1</sub> [methanol-PBS (55:45 v:v) solution] using the DPV method. The reduction peak currents (both at -650 E/mv and -250 E/mv (vs. Ag, AgCl (1M KCl))) have shown a linear relationship against the concentrations of AFB<sub>1</sub>. The DPV diagram of enzyme-MWNTs electrode response towards various AFB<sub>1</sub> concentrations is shown as (b)

been assembled. The mixture of anti-AFB<sub>1</sub> antibody and detoxifying enzyme was immobilized on the MWNTs-modified electrode. To optimize the MWNTs based enzyme: antibody immobilization parameters, the selection of the dilution of antibody and the ratio of enzyme/Ab used was studied in order to obtain the highest sensitivity. From the results, shown in Fig. 3 (a) and (b) 1:80 (v:v) dilution of Ab and 1:5 (v:v) of enzyme: Ab (higher enzyme ratio can widen the peak) has been chosen. Using this electrode, the CV of a 1.28μM AFB<sub>1</sub> solution and its solvent control were measured. The cyclic voltammogram is shown in Fig. 3 (c). Similarly to the enzyme-MWNTs

electrode, the enzyme-Ab-MWNTs electrode detected a reduction peak at  $-650$  E/mV. The pair of redox peaks also appeared, but was weaker than the peak at  $-650$  E/mV. The peak at  $-650$  E/mV was chosen for the quantitative analysis.



**Fig. 3 The current response of enzyme-Ab-MWNTs electrode**

The current response with AFB<sub>1</sub> solution with various Ab dilutions (v:v) (a) and various ratios of Ab:enzyme (v:v) (b) used during the electrode assembly. (c) Shows the CV (Cyclic voltammetry) diagram of enzyme-Ab-MWNTs electrode to A:  $1.28\mu\text{M}$  AFB<sub>1</sub> solution and B: Methanol-PBS control

**2.2.2 The dependence of response current to AFB<sub>1</sub> concentration with the enzyme-Ab-MWNTs electrode** Fig. 4 (a) shows the enzyme-Ab-MWNTs electrode response currents to various concentrations of AFB<sub>1</sub>. When measured at  $-650$  E/mV, between the AFB<sub>1</sub> concentrations ranged from  $0.016\mu\text{M}$  to  $0.48\mu\text{M}$ , the linear relationship was:

$I/\mu\text{A} = 4.811C/\mu\text{M} + 1.664$  ( $R^2 = 0.9957$ ), (DPV was used here), [shown as Fig. 4 (b)].

And between the AFB<sub>1</sub> concentrations ranged from  $0.40\mu\text{M}$  to  $2.4\mu\text{M}$  the linear relationship was:

$I/\mu\text{A} = 7.145C/\mu\text{M} + 1.844$  ( $R^2 = 0.9587$ ), (DPV was used here), [shown as Fig. 4 (c)].

The detection limit was found to be  $0.016\mu\text{M}$ . Compared the response currents from the Ab-Enzyme-MWNT electrode with the currents from enzyme-MWNTs electrode, the quantitative assay linear relationship ranges were widened and the sensitivity was improved for the antibody enhanced electrode.

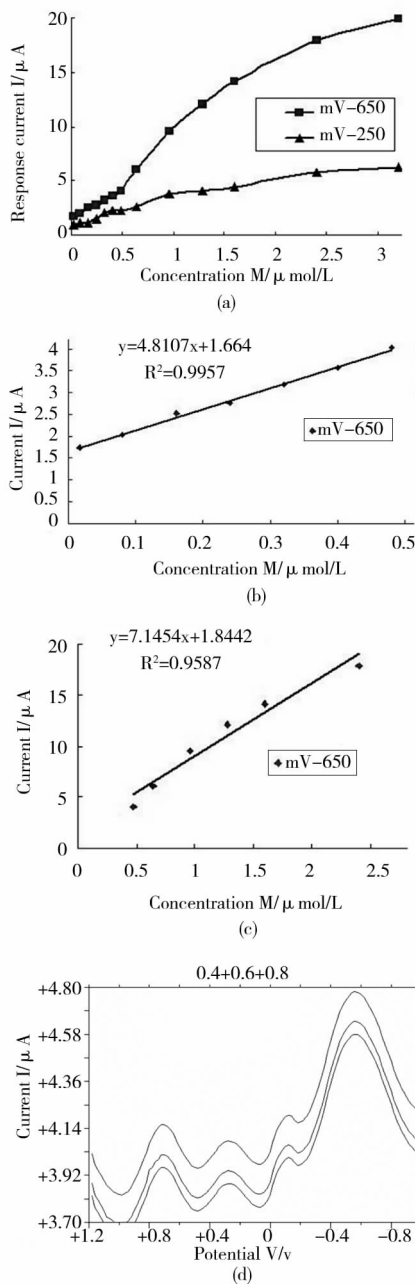
The DPV characters of the Enzyme-Ab-MWNT electrode at various AFB<sub>1</sub> concentrations are shown as Fig. 4 (d).

### 2.3 The reusability of the newly constructed Enzyme-Ab-MWNT electrode

Observation of the enzymatic dissociation of antigen was carried out by measuring the signals of  $0.064\mu\text{M}$  AFB<sub>1</sub> alternating with solvent control. When measuring the signals of the solvent control following each AFB<sub>1</sub> measurement, the zero response current indicated satisfactory dissociation of the AFB<sub>1</sub> analyte (regeneration function). This study shows the reusability of the electrode. Fig. 5 indicates a good reusability up to twenty times without any extra desorption process.

## 3 Discussion

There has been a strong preference for MWNTs in the assemblage of enzyme biosensors in concerned with their unique physical and chemical characteristics and considerable surface area which can increase the quantities of enzymes immobilized, multiply the reaction sites for substrates, and enhance the electrical conductivity and biosensor response<sup>[13-16]</sup>. The high surface area possessing multiple acidic sites may offer special opportunities for the immobilization of enzymes. It was confirmed that acid-treatment could enhance the functional carbonyl oxygen on MWNTs surface and could significantly improve the electron transfer kinetics<sup>[17-19]</sup>. In this study, acid-treated MWNTs were used as matrix for the combination of anti-AFB<sub>1</sub> antibody (Ab) and AFB<sub>1</sub> detoxifying enzyme by way

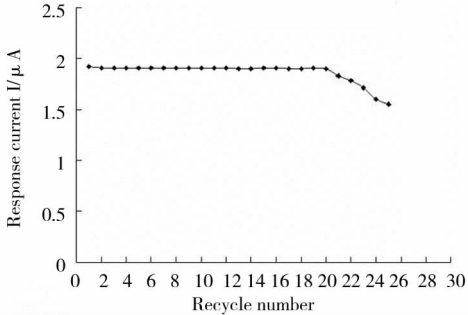


**Fig. 4 The enzyme-Ab-MWNTs electrode's DPV current response on various conc. of AFB<sub>1</sub>**

The DPV (Different pulse voltammetry) current response peaks (-650 E/mV (vs. Ag, AgCl (1M KCl)) on various conc. of AFB<sub>1</sub> (enzyme-Ab-MWNTs electrode) (a) The linear relationship in the concentration of AFB<sub>1</sub> range from 0.016μM to 0.48μM has shown as (b) and the range from 0.40μM to 2.4μM shown as (c). (d) Shows the DPV characters of the enzyme-Ab-MWNTs electrode's response on various conc. of AFB<sub>1</sub>

of diimide-activated amidation under ambient conditions.

In the study, two methods were applied to assemble



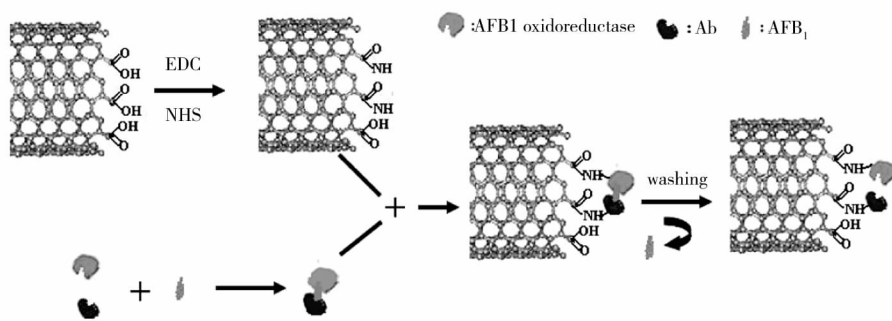
**Fig. 5 The reusability of the enzyme-Ab-MWNTs electrode**

The reusability of Ab-Enzyme-MWNT electrode was measured by measuring the response current of a 0.064 μM AFB<sub>1</sub> methanol-water (55:45, v: v) sample. Response currents to AFB<sub>1</sub> sample are constant up to 20-times of reuse

enzyme biosensor, one was immobilizing aflatoxin oxidoreductase on the electrode by covalent bonding of carboxylated Multi-Walled Carbon Nanotubes, and the other was immobilizing both aflatoxin oxidoreductase and specific anti-AFB<sub>1</sub> antibody on the electrode in the same way. In the second method, the strategy of substrate protection was used (illustrated in Fig. 6). This two kinds of biosensor were used in AFB<sub>1</sub> detection, and the results were as below. The character potential of AFB<sub>1</sub> on enzyme-MWNT modified electrode was at +400 E/mV and -250 E/mV (a pair of red-ox peaks), -650 E/mV (a reduction peak) in 1.28μM AFB<sub>1</sub> methanol-PBS (v: v 55: 45) solution. The detection linear range was from 0.16μM to 3.2μM. (correlation coefficient was 0.9593) and the detection limit was 0.16μM AFB<sub>1</sub>. While the corresponding linear range of enzyme-Ab-MWNT modified electrode was from 0.016μM to 0.48μM (correlation coefficient was 0.9957) and from 0.40μM to 2.4μM (correlation coefficient was 0.9587) with an improved detection limit of 0.016μM AFB<sub>1</sub>. The results indicated; the sensitivity of the aflatoxin oxidoreductase biosensor assembled by immobilized the enzyme and specific anti-AFB<sub>1</sub> antibody with MWNT under substrate protection strategy is 10 times higher than that of the enzyme-MWNT biosensor. According to these results, we supposed that there would be two possibilities in the second electrode assemblage method that led to higher sensitivity, the first

one was, the Enzyme-Ab-MWNT micro-system on the electrode functioned almost as an antibody coupled with canalization, could not only specifically bind the substrate like antibody but convert the substrate as enzyme, and this can occurred multi-times. Anti-AFB<sub>1</sub> antibody involved in this micro-system was full-antigen AFB<sub>1</sub> type I as illustrated in Fig. 7, this specific antibody bound the coumarin part of AFB<sub>1</sub> (furan-ring, the other side of AFB<sub>1</sub> was the binding site of the oxidoreductase). To support this explanation, the following experiment had been carried out previously<sup>[20]</sup>: After AFB<sub>1</sub> was absorbed by the specific anti-AFB<sub>1</sub> antibody (type I) coated on the microplate, the oxidoreductase was added in the micropores and treated with warm bath, at last the result showed that the amount of AFB<sub>1</sub> absorbed by the antibody in the test group was less than that of the control group. This suggested that AFB<sub>1</sub> absorbed by the antibody could also be catalyzed by the oxidoreductase. Since the enzyme-Ab microsystem had been constructed on the surface of the electrode, in the detection solution of lower concentration, the specific

AFB<sub>1</sub> antibody may play a role of enriching AFB<sub>1</sub> for ADTZ to catalyze. It was these AFB<sub>1</sub> enriched by antibody and catalyzed by the oxidoreductase that improved the electrode's sensitivity. The second possibility was, the method of constructing electrode by immobilizing antibody, aflatoxin oxidoreductase with AFB<sub>1</sub> existence has made the spatial distribution of the oxidoreductase on the electrode surface become more prone to the state benefiting the enzyme and substrate binding (Fig. 6). Compared with the method of immobilizing enzyme only, the enzyme-Ab microsystem has decreased stereospecific blockade between enzyme molecules, and increased the probability of enzyme and substrate binding, this may improve the sensitivity of the enzyme-Ab-MWNTs electrode. Of course, the detailed mechanisms of the enzyme-Ab-MWNTs electrode still need further researches. The work of this study demonstrated once again that the newly developing method of enzyme electrode construction was an effective way to improve the sensitivity of enzyme biosensor.



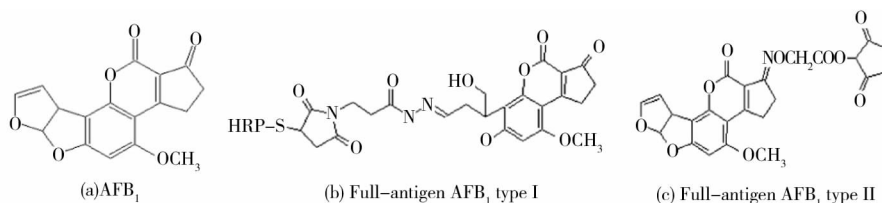
**Fig. 6 The illustration of the assembly enzyme-Ab-MWNTs electrode**

The illustration for how to immobilize the detoxifying enzyme and Ab on MWNTs modified electrode surface via AFB<sub>1</sub> directional location. The treated-MWNTs' connected with protein (Ab and enzyme) via acid amide groups. The proportion of "right position with right conformation" of enzyme with Ab can be increased by the existence of AFB<sub>1</sub>. AFB<sub>1</sub> was washed out by buffer before using

## 4 Conclusions

This paper introduced a new method to assemble Enzyme-Ab-MWNT biosensor which was potentially fit for Flow injection analysis (FIA) mode. The method was simple with immobilized Ab and enzyme on the electrode surface, combining the advantages of immune-affinity sensitivity with

an enzyme biosensor. The newly designed Enzyme-Ab-MWNT biosensor can detect AFB<sub>1</sub> at a concentration as low as 16 nM under methanol-water solution with the characteristics of continuous regeneration. The further improvement of this enzyme aflatoxin biosensor will focus on the detection limitation and its detection ranges. These works are carrying out now, and the newly development will published elsewhere.



**Fig. 7 The illustration of chemical structures of AFB<sub>1</sub>, and full-antigen AFB1 types**

(a) The illustration of the chemical structures of AFB<sub>1</sub> (b) The full-antigen AFB<sub>1</sub> type I (c) The full-antigen AFB<sub>1</sub> type II

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## 一种新的检测黄曲霉毒素 B<sub>1</sub> 的酶生物传感器的制作<sup>\*</sup>

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**摘要** 报道了一种新的检测黄曲霉毒素 B<sub>1</sub> 的生物传感器,该传感器以开管的多壁纳米碳管固定化黄曲霉毒素氧化还原酶制作传感电极检测黄曲霉毒素 B<sub>1</sub>,其线性范围达到 0.16 $\mu$ M-3.2 $\mu$ M,当把特异性的黄曲霉毒素 B<sub>1</sub> 抗体与黄曲霉毒素氧化还原酶通过多壁纳米碳管共固定化制作修饰电极,传感器的检测限提高到 16nM,灵敏度提高了 10 倍。用这种方法制作黄曲霉毒素酶生物传感器,使黄曲霉毒素酶生物传感器向实用化迈进了一步。

**关键词** 黄曲霉毒素 B<sub>1</sub> 酶电极 多壁碳纳米管

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