Enzyme-controlled dissolution of MnO₂ nanoflakes with enzyme cascade amplification for colorimetric immunoassay

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ABSTRACT

A new colorimetric immunosensing platform accompanying enzyme cascade amplification strategy was fabricated for quantitative screening of small-molecular mycotoxins (aflatoxin B₁, AFB₁ used in this case) coupling with enzyme-controlled dissolution of MnO₂ nanoflakes. The visual colored assay was executed by high-efficient MnO₂-3,3',5,5'-tetramethylbenzidine (TMB) system (blue). In the presence of ascorbic acid, MnO₂ nanoflakes were dissolved into Mn²⁺ ions, thus resulting in a perceptible color change from blue to colorless. The reaction could be weakened through ascorbate oxidase to catalyze ascorbic acid into dehydroascorbic acid, which indirectly depended on the concentration of ascorbate oxidase. By using ascorbate oxidase/anti-AFB₁ antibody-labeled gold nanoparticles, a novel competitive-type colorimetric enzyme immunoassay was developed for detection of AFB₁ on AFB₁-bovine serum albumin (BSA)-conjugated magnetic beads. Upon addition of target AFB₁, the analyte competed with the conjugated AFB₁-BSA on the magnetic beads for the labeled anti-AFB₁ antibody on the gold nanoparticles. Under optimal conditions, the absorbance decreased with increasing target AFB₁ within the dynamic range of 0.05 – 150 ng mL⁻¹ with a detection limit of 6.5 pg mL⁻¹ at the 3S_{blank} level. The precision and specificity of the MnO₂-TMB-based immunosensing system were acceptable. In addition, method accuracy was further validated for monitoring spiked peanut samples, giving results matched well with those obtained from commercialized AFB₁ ELISA kit.

KEYWORDS: MnO₂ nanoflakes; colorimetric immunoassay; aflatoxin B₁; enzyme cascade

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amplification

1. Introduction

Sensitive and specific determination of biotoxins including marine toxins and mycotoxins has been attracting great attention in the environmental pollution and food monitoring (Gawley et al., 2002; Bovee et al., 2011; Samdal et al., 2015; Nathanail et al., 2015; Lin et al., 2015a). A huge effort has been expanded in the field of simple and user-friendly assay development. Based on different signal-generation principles, colorimetric detection methods can be utilized to realize this purpose because of their simplicity, practicality and high-speed operation (Soh et al., 2015). Enzyme-based labelling strategies are usually used during this measurement (Berg et al., 2015; Geng et al., 2015; Qu et al., 2014). For example, horseradish peroxidase (HRP) could catalyze 3,3',5,5'-tetramethylbenzidine (TMB) for development of blue color in the presence of hydrogen peroxide (H_2O_2) (Chen et al., 2014). However, the detection sensitive was always limited due to the limitation of the labelling amount on each antibody. Moreover, the added H_2O_2 was often unstable and easily decomposed in air (Ray et al., 2014). Recent research has looked to develop innovative and powerful nanocatalysts with peroxidase-like activity (*e.g.*, Fe_3O_4 , Pt, Co_3O_4 , graphene oxide and bimetallic nano-alloys), controlling and tailoring their properties in a very predictable manner to meet the requirements of specific applications (Liang et al., 2013; Qin et al., 2014; Wu et al., 2014; Wang et al., 2014; Gao et al., 2015). A more preferable approach that can *in-situ* generate H_2O_2 and does not need the addition of H_2O_2 would be advantageous. Wang et al. utilized glucose oxidase to catalyze glucose for generation of H_2O_2 (Wang et al., 2015). Our group reported a simple colorimetric immunoassay by coupling with the TMB-Ag(I) system, in which Ag(I) could oxidize TMB directly in the absence of H_2O_2 (Lai et al., 2015). However, we recently found that the 1 : 1 oxidation in the TMB-Ag(I) system could be improved by using enzyme mimics, *e.g.*, nanocatalysts (Kumari et al., 2014; Su et al., 2012; Zhang et al., 2014; Gao et al., 2014).

To acquire a high sensitivity and a low detection limit, the signal amplification would be very crucial. Wang et al. utilized folate-polyoxometalate hybrid spheres as the oxidase mimics for the colorimetric immunoassay of human hepatocellular liver carcinoma cell line (Wang et al., 2011).

Perez's group employed ceria nanoparticles with oxidase-like activity as the labelled agents for colorimetric immunoassay (Asati et al., 2009). Manganese dioxide (MnO_2) is a material that has been applied in dry cell batteries, supercapacitors, ceramics, biosensors and organic synthesis (Wan et al., 2012). Moreover, MnO_2 exhibits huge potential prospect because of the abundance of manganese in the earth, low cost, low toxicity and the relative high activity. As is well known, MnO_2 nanostructures could catalyze TMB to produce a blue color in the absence of H_2O_2 . It has been reported that various-shaped nanostructures often exhibited different catalytic efficiencies (Wan et al., 2012; Liu et al., 2012). Inspiringly, the emergence of MnO_2 nanoflakes (< 100 nm, a kind of two-dimensional nanomaterials) opens a new horizon for advanced development of colorimetric assay (Wei et al., 2014). Meanwhile, we also noticed that some chemicals (*e.g.*, ascorbic acid and glutathione) could reduce MnO_2 to Mn^{2+} ion, which lost the peroxidase-like activity or oxidase-like activity (Zhai et al., 2014; Khan et al., 2005; Luo et al., 2004; Zhang et al., 2014), thereby resulting in the change in the color from blue to colorless in the presence of TMB. More favourably, ascorbic acid (AA) can be oxidized to dehydroascorbic acid (DAA) by ascorbate oxidase (AOx). To this end, our motivation in this work is to construct a new colorimetric detection system through enzyme-controlled MnO_2 -TMB system for development of colorimetric immunoassay with high sensitivity.

Aflatoxins, the highly toxic secondary metabolites produced by a number of different fungi, are present in a wide range of food and feed commodities, and are assumed significant because of their deleterious effects on human beings, poultry and livestock (Elzupir and Almer, 2014; Arduini et al., 2010; Lin et al., 2015b). The major aflatoxins of interest are designated B_1 , B_2 , G_1 and G_2 , however, aflatoxin B_1 (AFB_1) is usually predominant and most toxic. By using AFB_1 as a model analyte, we herein design a new MnO_2 -TMB-based colorimetric immunoassay for sensitive monitoring of AFB_1 on bovine serum albumin- AFB_1 (BSA- AFB_1)-modified magnetic beads by using AOx and anti- AFB_1 antibody-labeled nanogold particles as the competitors. With a competitive-type assay format, the carried AOx accompanying gold nanoparticles initially catalyzes AA to DAA, and decreases the dissolution of MnO_2 nanoflakes, thus resulting in a strong blue color and a high absorbance. The color and absorbance decreases with the increasing target AFB_1 in the sample. MnO_2 nanoflakes are not only used as the enzymatic mimics, but also utilized for progression of enzyme cascade reaction. The aim of this study is to

set up a high-efficient colorimetric immunoassay without the participation of H_2O_2 during the measurement.

2. Experimental

2.1. Material and chemicals

Polyclonal anti-AFB₁ antibody produced in rabbit (product no.: A8679, fractionated antiserum buffered aqueous solution) (Immunogen: aflatoxin B₁-KLH; Physical form: 0.01 M pH 7.4 phosphate-buffered saline solution containing 15 mM sodium azide), AFB₁ standards from *Aspergillus flavus* (product no.: A6636), 3,3',5,5'-tetramethylbenzidine (TMB), ascorbate oxidase (AOx), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Shanghai, China). AFB₁-BSA conjugate was obtained from the School of Food Science and Technology, Jiangnan University (Wuxi, China). Cetyltrimethylammonium bromide (CTAB) was achieved from Genview (USA). L-Ascorbic acid (AA) was acquired from Beijing Dingguo Biotechnol. Inc. (Beijing, China). Potassium permanganate (KMnO_4) was gotten from Fuchen Chemicals (Tianjin, China). All other reagents were used as received without further purification. All water used was the Milli-Q ultrapure grade (EMD Millipore). A pH 9.6 carbonate buffer solution was prepared by using 1.59 g Na_2CO_3 and 2.93 g NaHCO_3 in 1000 mL water. A pH 6.5 Tris-HCl buffer (4.0 mM) was prepared by adding the corresponding chemicals in 100 mL distilled water with the aid of 0.1 M HCl. 0.1 M citrate acid and 0.2 M Na_2HPO_4 were mixed and adjusted to pH 4.0 as the TMB substrate solution. A pH 7.4 PBS was prepared by using 2.9 g Na_2HPO_4 , 0.24 g KH_2PO_4 , 0.2 g KCl and 8.0 g NaCl in 1000 mL distilled water. The washing and blocking buffer were obtained by adding 0.05% Tween 20 (v/v) and 0.1 wt % BSA in PBS, respectively.

2.2. Synthesis of two-dimensional MnO_2 nanoflakes

MnO_2 nanoflakes were synthesized by using a typical hot-injection method according to this literature with minor modification (Wei et al., 2014). Before synthesis, two solutions including 0.3644 g CTAB and 0.0316 g KMnO_4 in 10 mL ultrapure water were prepared, respectively. And then, CTAB aqueous solution was heated to 140 °C in the oil bath under vigorous stirring.

Following that, the above-prepared KMnO_4 aqueous solution was quickly added into CTAB solution, and the temperature was maintained at $140\text{ }^\circ\text{C}$ for 2 h. Finally, the resulting suspension was centrifuged (three times, 12,000 g, 10 min) and washed with acetone. The obtained MnO_2 nanoflakes were re-dispersed into 1.0-mL distilled water for further use.

2.3. Monitoring of AOX activity based on MnO_2 -to- Mn(II) conversion with MnO_2 -TMB system

To monitor the AOX activity, $10\text{ }\mu\text{L}$ of AOX standard with different concentrations from 0 to 2000 mU mL^{-1} was initially mixed with $50\text{ }\mu\text{L}$ of $300\text{ }\mu\text{M}$ AA in pH 6.5 Tris-HCl buffer, and incubated for 15 min at room temperature. Following that, $50\text{ }\mu\text{L}$ of the above-prepared MnO_2 nanoflakes was injected into the mixture and reacted for 2 min. Afterwards, $100\text{ }\mu\text{L}$ of TMB substrate solution (1.0 mM , ethanol : pH 4.0 Tris-HCl buffer = 1 : 9) was added to the cell and reacted for 1.0 min at room temperature. Finally, a microplate reader (Tecan Infinite 200 Pro, TECAN, Switzerland) was used for monitoring of the resultant solution, and the absorbance was recorded at $\lambda = 652\text{ nm}$.

Scheme 1. Schematic illustration of MnO_2 nanoflakes-mediated colorimetric immunoassay on bovine serum albumin- AFB_1 (BSA-AFB_1)-modified magnetic beads by using AOX/anti- AFB_1 antibody-labeled nanogold particles with enzyme cascade amplification strategy and the MnO_2 -TMB system.

2.4. *MnO₂-TMB-based colorimetric immunoassay toward target AFB₁*

Scheme 1 gives schematic illustration of MnO₂-TMB-based colorimetric immunoassay. The preparation procedure of AFB₁-BSA-conjugated magnetic beads (designated as AFB₁-MB) and AOx/anti-AFB₁-labeled gold nanoparticles (designated as Ab-AuNP-AOx) were described in detail in our previous report with minor modification (Lai et al., 2015). The assay toward target AFB₁ was carried out as follows. Initially, 25 μ L of AFB₁ standard or sample and 50 μ L of Ab-AuNP-AOx ($C_{[Au]} \approx 38.8$ nM) were added in turn into 25 μ L of AFB₁-MB (10 mg mL⁻¹) in a 200- μ L PCR tube. Then the mixture was incubated for 60 min at 37 °C with gentle shaking. After being separated by an external magnet and washed with the washing buffer, 50 μ L of AA (300 μ M) in pH 6.5 Tris-HCl buffer was injected into the precipitate and incubated for 15 min at room temperature. After that, 50 μ L of the above-prepared MnO₂ nanoflakes was thrown to the mixture and reacted for 2 min. Finally, TMB (100 μ L, 1.0 mM) substrate solution was added in the mixture, and the absorbance was recorded as above.

3. Results and discussion

3.1. *Characterization of MnO₂ nanoflakes and MnO₂-TMB-based colorimetric assay*

In this work, MnO₂ nanoflakes were synthesized by a typical hot-injection method on the basis of reaction between KMnO₄ and CTAB at 140 °C. During the experiment, we observed that MnO₂ nanoflakes were formed immediately upon addition of KMnO₄ into CTAB solution. Meanwhile, the color of the mixture was changed from violet to brown. Figs. 1A-B show typical transmission electron microscope (TEM, FEI Titan 80-300, Hillsboro, USA) images of the synthesized MnO₂ nanostructures. Obviously, the morphology of the nanomaterials was planar sheet-like and ultrathin (Fig. 1B), indicating that the nanostructures were neither nanoparticles nor nanotubes. Such a two-dimensional (2D) nanoflakes structure could provide a large surface area for the reaction with TMB. As seen from Fig. 1A, the average size of the as-prepared MnO₂ nanoflakes was ~ 70 nm. Logically, one important question arises to whether the as-synthesized MnO₂ nanoflakes could be used as enzyme mimics to catalyze TMB in the absence of H₂O₂. To demonstrate this issue, 1.0 mM TMB (used as an example) was employed for direct incubation

with MnO₂ nanoflakes with different concentrations. The obtained product was monitored by using a microplate reader. As shown in Fig. 1C, the absorbance increased with the increasing concentration of MnO₂ nanoflakes. The results indicated that the as-prepared MnO₂ nanoflakes could catalyze the TMB to produce a visual color and cause the change in the absorbance in the absence of H₂O₂.

Fig. 1. (A,B) TEM images of the as-synthesized MnO₂ nanoflakes; (C) dependence of 1.0 mM TMB in the absorbance intensity on different-concentration MnO₂ nanoflakes; and (D) comparison of 1.0 mM TMB in the absorbance intensity between MnO₂-TMB system and AgNO₃-TMB system towards different-concentration (a) MnO₂ nanoflakes and (b) AgNO₃.

To further elucidate the advantages of the as-synthesized MnO₂ nanoflakes in the MnO₂-TMB system, a comparative study with our previously reported system (*i.e.*, Ag⁺-TMB system) (Lai et al., 2015) was carried out by assaying the same-concentration MnO₂ or AgNO₃ toward the change of 1.0 mM TMB in the absorbance intensity (Fig. 1D). As seen from Fig. 1D, the MnO₂-TMB system could exhibit a higher sensitivity and stronger absorbance intensity relative

to the Ag^+ -TMB system. That is to say, introduction of MnO_2 nanoflakes could display strong catalytic efficiency toward the same-concentration TMB, which provided a precondition for the development of highly sensitive colorimetric assay.

Fig. 2. UV-vis absorption spectra of (A-a) MnO_2 nanoflakes, (A-b) TMB, (A-c) MnO_2 nanoflakes + TMB, (B-a) TMB, (B-b) TMB + Mn(II) , (C-a) MnO_2 nanoflakes + TMB, and (C-b) AA + MnO_2 nanoflakes + TMB (Insets: the corresponding photographs) (100 μL of 1.0 mM TMB and 50 μL of the above-prepared MnO_2 nanoflakes used in the case).

To ensure the feasibility of MnO_2 nanoflakes-based colorimetric assay, some control tests were carried out. Recent reports have found that MnO_2 could catalyze TMB, and produce a blue color with an absorption peak at 652 nm. As shown from curves 'a-b' in Fig. 2A, no characteristic absorption peaks were observed at MnO_2 nanoflakes (curve 'a') or TMB (curve 'b') alone. After mixture of TMB with MnO_2 nanoflakes, two strong characteristic peaks were appeared (Fig. 2A, curve 'c'). Moreover, the color of the solution was changed from colorless to blue (Fig. 2A, photograph 'c'), indicating the catalytic ability of MnO_2 nanoflakes toward TMB.

Maybe, we might be asked whether Mn(II) could catalyze TMB. Fig. 2B shows the UV-vis adsorption spectra of TMB solution in the absence and presence of Mn(II) . No matter whether Mn(II) was added into the solution or not, no absorption peaks were achieved (Fig. 2B, curve 'a'

vs. curve 'b'). Meanwhile, the color of TMB solution was not changed (Fig. 2B, photographs 'a-b'). The result indicated that Mn(II) could not catalyze the TMB for the development of colorimetric assay.

Furthermore, we also investigated the effect of ascorbic acid on the MnO₂-TMB system, *i.e.*, whether the added ascorbic acid could weaken the MnO₂-TMB system to generate the visual color. Two strong characteristic peaks (curve 'a') and a thick blue color (photograph 'a') could be achieved when mixture of TMB with MnO₂ nanoflakes (Fig. 2C). When MnO₂ nanoflakes were first reacted with excess ascorbic acid, however, the subsequently added TMB did not cause appearance of blue color (Fig. 2C, photograph 'b'). Meanwhile, two absorption peaks for the MnO₂-TMB system were disappeared (Fig. 2C, curve 'b'). The reason might be ascribed to the fact that MnO₂ nanoflakes in the detection solution were dissolved into Mn(II) by AA. Hence, the results revealed that the MnO₂-TMB colorimetric detection system could be weakened by AA. Significantly, the residual amount of AA in the same could be controlled through AOX toward the oxidation of AA. In other words, the MnO₂-TMB system could be used for the monitoring of AOX activity.

3.2. Optimization of experimental conditions

To acquire an optimal analytical performance, some experimental conditions influencing the analytical properties of the MnO₂-TMB system should be studied. In this work, the visual color derived from the reaction between TMB and MnO₂ nanoflakes. Fig. 3A shows the dynamic response curve of the MnO₂-TMB reaction relative reaction time. The reaction was very fast, and reached to the steady-state equilibrium within one minute. To avoid possible error resulting from different additions of TMB and deduct the responses induced by various-substrate reaction, the signal was recorded from 30 s (after the addition of sample) until equilibrium was reached, ~ 1.0 min, for the colorimetric development.

Normally, ascorbic acid can reduce MnO₂ nanoflakes to Mn(II) ions, thereby inhibiting the development of the MnO₂-TMB system in the color and decreasing the absorbance of the system. To this end, we investigated the responses of the MnO₂-TMB system (100 μ L of 1.0 mM TMB and 50 μ L of the above-prepared MnO₂ nanoflakes used in the case) toward ascorbic acid

standards with different concentrations. As shown in Fig. 3B, the absorbance decreased with the increasing AA concentration, and tended to level off after 300 μM . An obvious jumping plot could be observed at 300 μM AA. That it to say, the change in the absorbance was more obvious with the decreasing AA concentration at $\leq 300 \mu\text{M}$ than that of high-concentration ascorbic acid. Considering development of the following MnO_2 -TMB-based immunoassay, 300 μM AA should be preferable in this work.

Fig. 3. Effects of (A) reaction time between TMB and MnO_2 nanoflakes and (B) concentration of ascorbic acid on the absorbance of the MnO_2 -TMB system (*Inset B*: Linear plots); (C) UV-vis absorption spectra of (a) AOx + AA + MnO_2 + TMB, (b) AOx + MnO_2 + TMB, (c) AA + MnO_2 + TMB, (d) AOx + MnO_2 and (e) AOx + TMB, respectively (*Insets*: The corresponding photographs for figure 'C'); and (D) the responses of the MnO_2 -TMB system toward different-activity AOx (100 μL of 1.0 mM TMB, 50 μL of the above-prepared MnO_2 nanoflakes and 300 μM AA used in the case).

3.3. Control tests and monitoring of enzymatic activity with the MnO_2 -TMB system

To further monitoring whether the signal of the MnO₂-TMB system could be really realized by AOX-controlling AA amount, several control tests were carried out under different conditions (Fig. 3C). As indicated from curve 'c' in Fig. 2A, it has been demonstrated that two characteristic absorption peaks and a strong blue color could be observed in the presence of MnO₂ nanoflakes and TMB. As seen from curve 'a' and photograph 'a' in Fig. 3C, introduction of AOX and AA in the MnO₂-TMB system was not almost changed the characteristic absorption peaks and visual color. When only AA was present in the MnO₂-TMB system, however, the absorption peaks were disappeared (curve 'c') and the solution became colorless (photograph 'c') in Fig. 3C. The results indicated that the added AA could be consumed by AOX, thus displaying the inherent color of the MnO₂-TMB system. Moreover, the presence of AOX could not cause the significant change in the absorbance and visual color of the MnO₂-TMB system (Fig. 3C, curve 'b' and photograph 'b'). Meanwhile, we also observed that MnO₂ nanoflakes (curve 'd' and photograph 'd') or TMB (curve 'e' and photograph 'e') alone could not react with AOX to induce development of the absorbance and blue (Fig. 3C). Comparison curve/photograph 'a' with curve/photograph 'c', the MnO₂-TMB system could be controlled by AOX in the presence of AA. Furthermore, we utilized the MnO₂-TMB system for the detection of AOX activity by using AA as the enzymatic substrate. The assay principle was schematically illustrated in Scheme 1. As seen from Fig. 3D, the absorbance increased with the increasing AOX activity, indicating that the system could be employed for quantitative monitoring of AOX activity. Favorably, a low detection limit could be estimated to $\sim 12 \text{ mU mL}^{-1}$ AOX at the $3s_{\text{blank}}$ criterion. The results also suggested that the absorbance of using the MnO₂-TMB system could be significantly changed only if the level of AOX in the sample was lower than 14 mU mL^{-1} , which also provide a vital precondition for the development of high-efficient MnO₂-TMB-based colorimetric immunoassay.

3.4. Analytical performance of MnO₂-TMB-based colorimetric immunoassay

To investigate the applicable potential of the developed MnO₂-TMB visual system on the colorimetric immunoassay, AOX/anti-AFB₁-labeled gold nanoparticles (Ab-AuNP-AOX) was utilized for the detection of target AFB₁ on AFB₁-BSA-conjugated magnetic beads (AFB₁-MB) with a competitive-type immunoassay format (Scheme 1). In the presence of target AFB₁, the

analyte competed with the conjugated AFB₁-BSA on magnetic beads for the labeled anti-AFB₁ antibody on gold nanoparticles. The conjugated amount of Ab-AuNP-AOx on the AFB₁-MB decreased with the increasing target AFB₁ in the sample, which was collected by using an external magnet. In this case, the carried AOx accompanying gold nanoparticles decreased, which catalyzed relatively less AA molecules, thus decreasing the reaction efficiency of ascorbic acid toward the MnO₂-TMB system. Under optimal conditions, the absorbance decreased with the increasing target AFB₁ in the sample (Fig. 4A). A good linear dependence between the absorbance and AFB₁ concentration was achieved within the dynamic working range from 0.05 to 150 ng mL⁻¹ with a detection limit (LOD) of 6.5 pg mL⁻¹ on the basis of 3s_{blank} criterion (Fig. 4A, inset). The linear regression equation could be fitted to $y = -0.0065 \times C_{[AFB_1]} + 1.0561$ (ng mL⁻¹, $R^2 = 0.9973$, $n = 21$). Each data point represented the average value obtained from three different measurements. The maximum relative standard deviation (RSD) was 9.4%. Further, we studied the batch-to-batch reproducibility of MnO₂-TMB-based immunoassay toward three level including 0.1, 20 and 100 ng mL⁻¹ AFB₁, and the RSDs were 6.1%, 9.2% and 7.9% ($n = 3$), respectively. These results suggested a good reproducibility and precision of the colorimetric immunoassay. Inspiringly, the LOD of our design was obviously even lower compared to the existed commercialized AFB₁ ELISA kits (*e.g.*, Quicking Biotech: 100 ppt; MaxSignal®: 50 pg mL⁻¹; MyBioSource: 250 pg mL⁻¹; Diagnostic Automation Inc.: 5.0 pg mL⁻¹). Owing to the legal limit of AFB₁ in foodstuff (< 2.0 ng mL⁻¹), the developed colorimetric immunoassay coupling with the MnO₂-TMB system can completely meet the need of AFB₁ detection in foodstuff.

To further monitor the specificity of MnO₂-TMB-based colorimetric immunoassay toward target AFB₁, the system was employed for challenging other mycotoxins [*e.g.*, AFB₂, AFG₁, AFG₂ and ochratoxin A (OTA)] and marine toxins [*e.g.*, okadaic acid (OA)]. As shown from Fig. 5B, the MnO₂-TMB-based colorimetric immunoassay displayed a high cross-reactivity toward AFB₂, whereas no false compliant results were achieved for AFG₁, AFG₂ OTA and OA. The reason might be attributed to the fact that anti-AFB₁ antibody used in this work has a high cross-reactivity with AFB₂, thus resulting in the change in the absorbance. These results clearly revealed that the specificity of MnO₂-TMB-based colorimetric immunoassay is acceptable.

Fig. 5. (A) UV-vis absorption spectra of MnO₂-TMB-based colorimetric immunoassay toward AFB₁ standards with different concentrations (ng mL⁻¹) (*Inset*: The corresponding linear curve), and (B) the specificity of the developed immunoassay against target AFB₁, AFB₂, AFG₁, AFG₂, OTA and OA (50 ng mL⁻¹ used in this case).

3.5. Monitoring of real peanut samples

The feasibility of applying the developed colorimetric immunoassay to evaluate AFB₁ levels in a complex matrix was also monitored by spiking AFB₁ standards in the peanut samples. The extraction and spiking procedure were described in detail in our previous work (Lin et al., 2015b). To verify the accuracy of MnO₂-TMB-based colorimetric immunoassay, the assayed results toward these peanut samples were compared with those obtained from commercialized AFB₁ ELISA kit (Diagnostic, Automation Inc.). The data are listed in Table 1. Statistical comparison between two methods was performed by using a *t*-test for comparison of means preceded by the application of an *F*-test. The *t*-test statistics for each sample was calculated by using independent two-sample *t*-test with equal sample sizes and equal variance. As seen from Table 1, no significant differences were encountered between the two methods at the 0.05 significance level because the t_{exp} were in all cases below than t_{crit} ($t_{\text{crit}[4,0.05]} = 2.77$). the regression equation between two methods could be fitted to $y = 1.0017x + 0.3017$ ($R^2 = 0.9978$, $n = 30$). The slope of the equation for the average values obtained between two methods was close to the ideal. Thus, the MnO₂-TMB-based colorimetric immunoassay could be utilized for quantitative monitoring of target AFB₁ in the complex systems.

4. Conclusions

In this work, we found a new colorimetric detection system on the basis of conventional TMB substrate by using MnO_2 nanoflakes as the enzymatic mimics. The system was carried out by enzyme-catalyzed ascorbic acid to weaken the development of MnO_2 -TMB in the visual color. The color and density in the absorbance could be controlled by conversion of MnO_2 nanoflakes to Mn(II) . Compared with classical TMB- H_2O_2 -based detection system, highlights of our strategy can be simply summarized as follows: (i) The method can efficiently avoid the participation of H_2O_2 during this measurement; (ii) natural enzyme (*i.e.*, ascorbate oxidase) and enzymatic mimic (*i.e.*, MnO_2 nanoflakes) were utilized for the amplification of detectable signal coupling with enzyme cascade reaction-based amplification strategy; and (iii) Two-dimensional MnO_2 nanoflakes can exhibit higher surface area for the oxidation of TMB. Importantly, the MnO_2 -TMB system could be utilized for the development of colorimetric immunoassay with high sensitivity, good reproducibility and acceptable method accuracy with commercialized ELISA kit. Nevertheless, only one disadvantage of the developed strategy involved in the multi-step operations, which might make the immunoassay complicated and time-consuming though MnO_2 nanoflakes could simplify the colorimetric reaction system (*i.e.*, the MnO_2 -TMB system). To fulfill the potential application for point-of-care testing, future work should focus on the improvement of operation steps.

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Table 1. Results obtained by the MnO₂-TMB-based colorimetric immunoassay and commercial AFB₁ ELISA Kit (Diagnostic Automation Inc., LOD = 5.0 pg mL⁻¹) for spiked peanut samples

sample	method; concentration (ng mL ⁻¹ , mean ± SD, <i>n</i> = 3) ^a		
no.	MnO ₂ -TMB-based immunoassay ^a	AFB ₁ ELISA kit	<i>t</i> _{exp}
1	1.7 ± 0.1	1.8 ± 0.2	0.77
2	13.2 ± 0.9	14.1 ± 0.6	1.44
3	60.1 ± 2.1	62.7 ± 1.5	1.74
4	93.7 ± 2.1	90.1 ± 1.9	2.20
5	124.2 ± 1.9	127.5 ± 2.3	1.92
6	74.5 ± 1.5	73.2 ± 0.9	1.29
7	0.89 ± 0.1	0.93 ± 0.09	0.51
8	23.5 ± 1.1	22.3 ± 0.8	1.53
9	51.2 ± 1.8	52.7 ± 1.2	1.20
10	5.4 ± 0.9	7.1 ± 0.4	2.40

^a Each sample was determined in triplicate, and the high-concentration AFB₁ sample was assayed with an appropriate dilution. The linear regression equation for these usable mean data between two methods is as follows: $y = 1.0017x + 0.3071$ ($R^2 = 0.9978$, $n = 30$; x axis: by MnO₂-TMB-based colorimetric immunoassay; y axis: by AFB₁ ELISA kit).