Composite Films of CdS Nanoparticles, MoS_2 Nanoflakes, Reduced Graphene Oxide, and Carbon Nanotubes for Ratiometric and Modular Immunosensing-Based Detection of Toxins in Cereals

Aori Qileng[1,2], Shule Huang[1], Liang He[1], Weiwei Qin[1], Weipeng Liu[1], Zhenlin Xu[2,*], Yingju Liu[1,3,*]

[1] Department of Applied Chemistry, College of Materials and Energy, South China Agricultural University, Guangzhou 510642, China

[2] The Guangdong Provincial Key Laboratory of Food Quality and Safety, College of Food Science, South China Agricultural University, Guangzhou 510642, China

[3] Guangdong Laboratory of Lingnan Modern Agriculture, Guangzhou 510642, China.

*Fax: 86-20-85285026, Phone: 86-20-85280319, E-mail: jallent@163.com (Z. Xu), liuyingju@hotmail.com (Y. Liu)

Experimental section

1. Chemicals

Most chemicals such as ascorbic acid (AA), $CdCl_2 \cdot {}_{5/2}H_2O$, $KMnO_4$, chloroform, thiourea, dodecylamine, ethylene glycol (EG), tetrahydrofuran, ($(NH4)_6Mo_7O_{24} \cdot 4H_2O$), L-cysteine, L-Cysteine monohydrochloride, NH_4Cl, polyvinylpyrrolidone (PVP, MW = 40000) were brought from Beijing Inno Chem Science & Technology Co. Ltd. Tween 20 was brought from Acros Organics. N-hydroxysuccinimide (NHS) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were supported by Beijing J&K Scientific Ltd. Graphite flake (200 mesh, 99.8%) was obtained from Alfa Aesar. Poly (isobutylene-alt-maleicanhydride) (Mw~6000 Da) was obtained from Sigma Aldrich. Carboxyl multi-wall carbon naotubes (CNT, No.XFM06) were brought from Nanjing XFNANO Materials Tech Co., Ltd. Nafion (5%) was supplied by Dupont. 3M™ copper conductive tape (double coated) was from Ted Pella, Inc. OTA, ABF_1, ZEN antibodies (1 mg/mL) and corresponding antigens (1 mg/mL) was from College of Food Sciences, South China Agricultural University, while secondary goat anti-rabbit antibody (Ab_2, 1 mg/mL) was from Santa Cruz. The ratio of KH_2PO_4 and Na_2HPO_4 was adjusted to prepare phosphate buffer solution (PBS, 1/15) with different pH values. PBST was prepared by adding Tween-20 (0.5%) to PBS (0.01 M, pH 7.4).

2. Preparation of CdS Nanoparticles/MoS_2 Nanoflakes/Reduced Graphene Oxide/ Carbon Nanotubes composite

Firstly, graphene oxide (GO) was synthesized from graphite flakes. In an ice bath, $KMnO_4$ (14 g) and graphite flakes (4 g) were slowly added into 160 mL of H_2SO_4 solution under slowly stirring. Then, it was mixed with ice water (400 mL) and stirred ceaselessly for 24 h at 35 °C. After that, H_2O_2 was added when the color of the solution didn't change. After stirring for 2 h, the solution was separated by centrifugation at 5000 rpm, and then washed with 300 mL of HCl solution until the pH value was 7.

Secondly, MoS_2 nanoflakes/reduced graphene oxide (MG) was fabricated by a one-step solvothermal method. After adding GO (25 mg) into 50 mL water, the solution was sonicatied for 1 h, followed by the gradual addition of L-cysteine (2.8 mmol) and

ammonium heptamolybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 0.1 mmol). When the pH of the solution was adjusted to 7 by HCl and stirred for 30 min, it was transferred to a 50-mL Teflon-lined stainless steel autoclave for 24 h at 200 °C. The precipitate was washed with ethanol and water at least four times.

Then, the flexible MoS_2 nanoflakes/reduced graphene oxide/carbon nanotubes (MGC) film was fabricated by vacuum filtration. Typically, CNT solutions were prepared by dispersing 70 mg of CNT and 120 μL of Nafion in 40 mL ethanol, sonicating for 30 min and settling statically for 4 h, while MG solution was prepared by adding 6.25 mg MG into 40 mL H_2O and sonicating for 30 min. After 4 mL of the MG solution was added and filtrated, the CNT solution and MG solution were alternately added and filtrated for 15 times. After freeze-drying in liquid nitrogen and vacuum freeze-drying, the film was peeled off as a MGC film and adhered to the copper conductive tape.

Finally, the chemical bath deposition was used to modify the CdS nanoparticles on the surface of the MGC film. Specifically, NH_4Cl (0.0076 mol), thiourea (0.18 mol), $CdCl_2$ (0.0013 mol) and ammonia solution (25.84 mL) were dissolved in 500 mL water. Then, the MGC film was added into the above solution at 80 °C for 20 min. After washing with water and drying, the CMGC film was obtained.

3. Synthesis of CuS@Ab_2 Label

In a typical synthesis of CuS, a solution was prepared by mixing 50 mL of EG, 0.3 g of PVP and 4 mmol of $Cu(NO_3)_2\cdot 3H_2O$. Then, it was maintained at 175 °C for 20 min under vigorously magnetic stirring. After 6 mmol thiourea was quickly injected, the mixture was transferred into a stainless-steel autoclave for 24 h at 160 °C. Finally, after washing with ethanol and water several times, the black precipitates were collected.

The amphiphilic polymer was synthesized according to the previous report [1]. Briefly, 1.542 g (10 mmol monomer) of poly(isobutylene-alt-maleic anhydride) were placed in a round flask. Then, 7.5 mmol dodecylamine was dissolved in 50 mL of anhydrous THF, transferred into the flask and stirred at 60 °C for 3 h. After concentrating the mixture to one-fifth of the original volume by a rotary evaporator, it was further stirred at 60 °C overnight. The obtained polymer was redissolved in $CHCl_3$ and the volume was adjusted to

around 12.5 mL. Then, 2 mL CuS nanoparticles solution (~ 4 mg/mL), 136 μL of amphiphilic polymer stock solution and 3 mL of $CHCl_3$ were mixed together and stirred for 20 min. After discarding the solvent, 6 mL of NaOH aqueous solution (0.1 M) was added and sonicated, thus a stable polymer was grafted on colloidal CuS NPs to form abundant carboxyl groups (CuS-COOH). Then, 8 mL water was added and the solution was purified by ultracentrifugation for 3 times. The obtained solution was further dialyzed against PBS for 2 days.

To form CuS@Ab_2, the above solution (2 mL) was centrifuged and dissolved in 2 mL NHS/EDC solution (10 mg mL^{-1}/20 mg mL^{-1}). Then, 10 μL Ab_2 (1 mg mL^{-1}) was added, incubated for 24 h and centrifuged, thus the CuS@Ab_2 label was obtained and redispersed in 1 mL of phosphate buffer (0.01 M, pH 7.4).

4. Analysis of Real Samples

Eight real samples (4 maize samples, No.1, 3, 5, 7 and 4 millet samples, No. 2, 4, 6, 8) were artificially infected by *Aspergillus flavus* (No. 3 and 4), *Penicillium verrucosum* (No. 5 and 6) and *Fusarium graminearum* (No. 7 and 8), which were bred on the potato dextrose agar medium and incubated for 15 days at 25 °C. Then, the samples of millet (10 g) and maize (10 g) were infected with strains and incubated at 25 °C during thirty day.

To extract AFB_1 from sample, 4 g of maize or millet were weighted and added into 20 mL mixture of acetonitrile and water (84:16, v/v). To extract OTA, 4 g of maize or millet and added into 20 mL mixture of acetonitrile, water and acetic acid (79:20:1, v/v/v). To extract ZEN, 4 g of maize or millet were weighted and added into 20 mL mixture of acetonitrile and water (90:10, v/v). All these samples were mixed by shaking for 30 s and then sonicating for 30 min. The filtrate was collected by using 0.22 μm nylon-66 membranes. Finally, the process of extraction was repeated twice. After diluting in proper times, the solution was used for HPLC-MS confirmation.

A C_{18} Gravity capillary column (150 mm×46 mm×2.7 μm, Agilent, USA) was used as chromatographic separations. For OTA and AFB_1, the mobile phase was prepared from solvent A (methanol) and solvent B (0.1% formic acid in water) at a ratio of 80% and 20% (A: B). The sample injection volume was 10 μL, while the flow rate was 0.5 mL min^{-1}. For

ZEN, the mobile phase was prepared from solvent A (methanol) and solvent B (0.1% formic acid in water) at a ratio of 90% and 10% (A: B). The sample injection volume was 10 μL, while the flow rate was 0.5 mL min^{-1}. The mass spectrometer equipped with an ESI source was performed in positive polarity and the parameters of MS were as follows: capillary temperature, 260 °C; spray voltage, 5.5 kV; tube lens, 70 V; sheath gas, nitrogen.

5. Materials Characterization

An electrochemical workstation (CHI660D, Chenhua Instruments Co. Ltd., China) and a photoelectrochemical system (PEAC 200A, Ida, China) using LED as an irradiation source (20 mW/cm^2) was used. A three-electrode system consisted of the modified tape-electrode (working electrode), the counter electrode (Pt electrode) and the auxiliary electrode (Ag/AgCl electrode). The morphology was detected by scanning electron microscopy (SEM, XL30-ESEM, EFI, Nederland) and transmission electron microscope (TEM, Tecnai12, EFI, Nederland). Energy dispersive spectrometer (EDS, SS550&SEDX-550, Shimadzu, Japan), X-ray photoelectron spectroscopy (XPS, Escalab 250Xi, America), and UV-vis spectrophotometer (UV2550, Shimadzu, Japan) were employed. High performance liquid chromatography-mass spectroscopy (HPLC-MS, API3200, AB SCIEX, America) were used to detect real samples.

6. Electrochemical Measurements

As shown in Scheme 1, the CMGC film was acted as the photoelectrode due to its high photocurrent, followed by using L-Cysteine monohydrochloride solution to decorate carboxyl groups for 6 h. Subsequently, the photoelectrode was thoroughly washed, followed by spreading EDC/NHS solution (20 mg mL^{-1}/10 mg mL^{-1}) on the surface to activate carboxyl groups at 20 °C for 1 h. Then, 20 μL of antigen (400-fold dilution by 0.01 M PBS, pH 7.4, 10 μg/mL) was decorated on the surface at 37 °C for 1 h. After washing, the excess active sites were blocked with 20 μL of blocking reagents at 37 °C for 1 h. Basing on the competitive format, a solution containing 10 μL sample at different concentrations and 10 μL antibody solution (400-fold dilution by 0.01 M PBST, 10 μg/mL) were further incubated on the electrode at 37 °C for 1 h. Then, the electrode was incubated with 20 μL of

the CuS@Ab_2 conjugate (2-fold dilution by 0.01 M PBS, pH 7.4) at 37 °C for 60 min. Immediately, the above electrode was used as the working electrode, while the PEC current was measured in PBS solution (0.1 M AA, pH 7.4, 1/15 M) on the PEC workstation. Then, the electrode was dried in N_2 atmosphere and CV was performed in 4 M KOH solution at a scan rate of 100 mV/s from -1.0 V to 0.5 V.

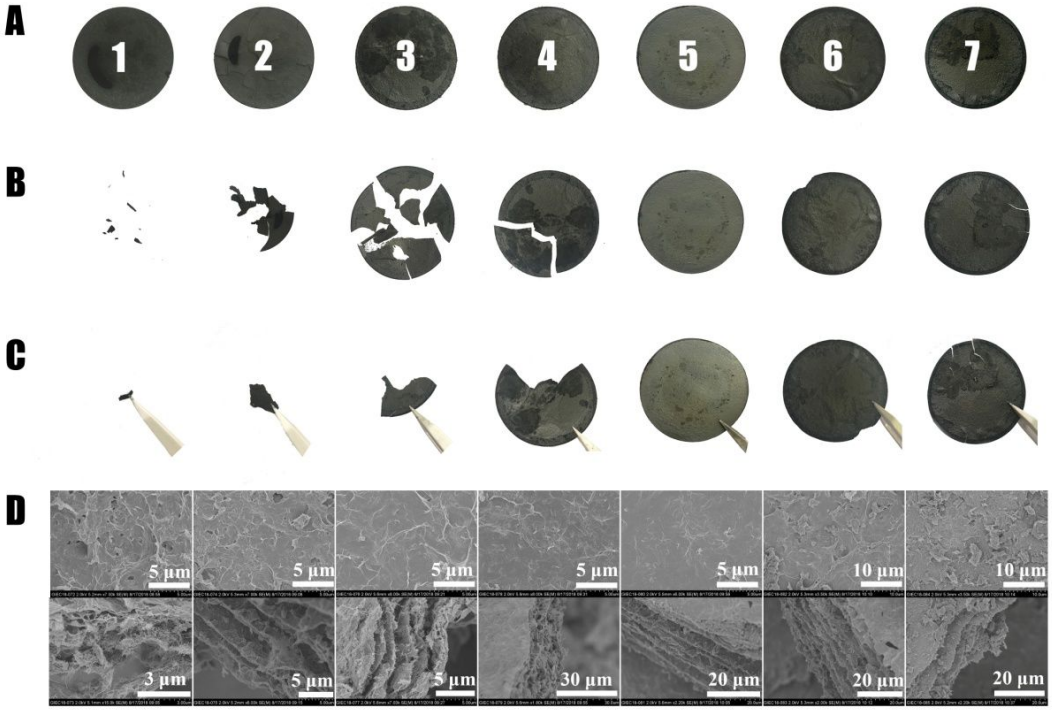

Fig. S1 The photographs of the films on the filter paper (A), after peeling from the filter paper (B) and after bending (C). (D) SEM of the films when they were prepared from CNT/Nafion with different CNTs including 30 mg (1), 40 mg (2), 50 mg (3), 60 mg (4), 70 mg (5), 80 mg (6) and 90 mg (7).

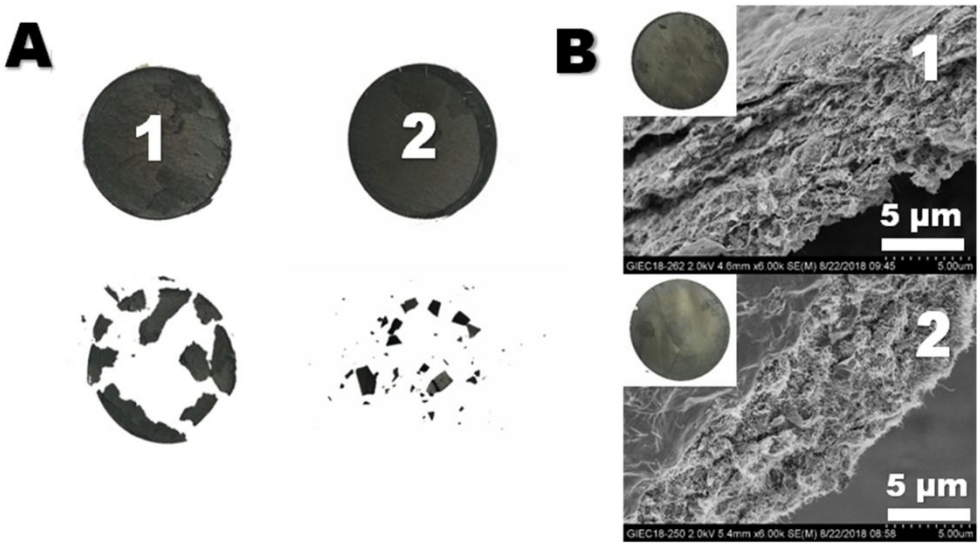

Fig. S2 The film was dried at room temperature after treating with liquid nitrogen (A1) or freeze-dried after the filtration (A2). B1 and B2 were the film from CNTs in different Nafion contents of 100 μL and 300 μL.

Table S1 The equations of I_{PEC} and I_{CV} for AFB_1, OTA, ZEN on different electrodes.

Target	Electrode	Signal	Relevant Equations	r^2
AFB_1	ITO	PEC	$I_{PEC} = 4.57 + 1.45\log C_{AFB1}$ (μg/L)	0.945
		CV	$I_{CV} = -0.10 - 1.72\log C_{AFB1}$ (μg/L)	0.975
	FTO	PEC	$I_{PEC} = 5.01 + 1.72\log C_{AFB1}$ (μg/L)	0.982
		CV	$I_{CV} = 0.21 - 2.98\log C_{AFB1}$ (μg/L)	0.976
	GCE	PEC	$I_{PEC} = 8.43 + 2.87\log C_{AFB1}$ (μg/L)	0.960
		CV	$I_{CV} = -0.80 - 3.59\log C_{AFB1}$ (μg/L)	0.929
OTA	ITO	PEC	$I_{PEC} = 4.10 + 1.27\log C_{OTA}$ (μg/L)	0.989
		CV	$I_{CV} = -0.07 - 2.82\log C_{OTA}$ (μg/L)	0.973
	FTO	PEC	$I_{PEC} = 5.30 + 1.59\log C_{OTA}$ (μg/L)	0.977
		CV	$I_{CV} = -0.05 - 3.07\log C_{OTA}$ (μg/L)	0.968
	GCE	PEC	$I_{PEC} = 6.09 + 1.93\log C_{OTA}$ (μg/L)	0.983
		CV	$I_{CV} = -0.71 - 3.93\log C_{OTA}$ (μg/L)	0.944
ZEN	ITO	PEC	$I_{PEC} = 4.36 + 1.43\log C_{ZEN}$ (μg/L)	0.961
		CV	$I_{CV} = 0.29 - 2.69\log C_{ZEN}$ (μg/L)	0.973
	FTO	PEC	$I_{PEC} = 4.60 + 1.47\log C_{ZEN}$ (μg/L)	0.939
		CV	$I_{CV} = -0.41 - 3.57\log C_{ZEN}$ (μg/L)	0.968
	GCE	PEC	$I_{PEC} = 6.36 + 2.17\log C_{ZEN}$ (μg/L)	0.968
		CV	$I_{CV} = -0.64 - 3.78\log C_{ZEN}$ (μg/L)	0.944

Table S2 The relationship between the logarithm of toxin concentrations and the logarithm of I_{PEC}/I_{CV} on different electrodes.

Target	Electrode	Relevant Equations	r^2
AFB_1	ITO	$\log (I_{PEC}/ I_{CV}) = 1.21 + 0.94\log C_{AFB1}$ (μg/L)	0.942
	FTO	$\log (I_{PEC}/ I_{CV}) = 0.94 + 0.92\log C_{AFB1}$ (μg/L)	0.983
	GCE	$\log (I_{PEC}/ I_{CV}) = 1.27 + 0.94\log C_{AFB1}$ (μg/L)	0.984
	SUM	$\log (I_{PEC}/ I_{CV}) = 1.14 + 0.93 \log C_{AFB1}$ (μg/L)	0.991
OTA	ITO	$\log (I_{PEC}/ I_{CV}) = 0.94 + 0.80 \log C_{OTA}$ (μg/L)	0.953
	FTO	$\log (I_{PEC}/ I_{CV}) = 1.02 + 0.79\log C_{OTA}$ (μg/L)	0.983
	GCE	$\log (I_{PEC}/ I_{CV}) = 1.01 + 0.80\log C_{OTA}$ (μg/L)	0.976
	SUM	$\log (I_{PEC}/ I_{CV}) = 1.01 + 0.79\log C_{OTA}$ (μg/L)	0.986
ZEN	ITO	$\log (I_{PEC}/ I_{CV}) = 1.23 + 0.95\log C_{ZEN}$ (μg/L)	0.953
	FTO	$\log (I_{PEC}/ I_{CV}) = 1.39 + 0.96\log C_{ZEN}$ (μg/L)	0.983
	GCE	$\log (I_{PEC}/ I_{CV}) = 1.14 + 0.97\log C_{ZEN}$ (μg/L)	0.976
	SUM	$\log (I_{PEC}/ I_{CV}) = 1.23 + 0.96\log C_{ZEN}$ (μg/L)	0.997

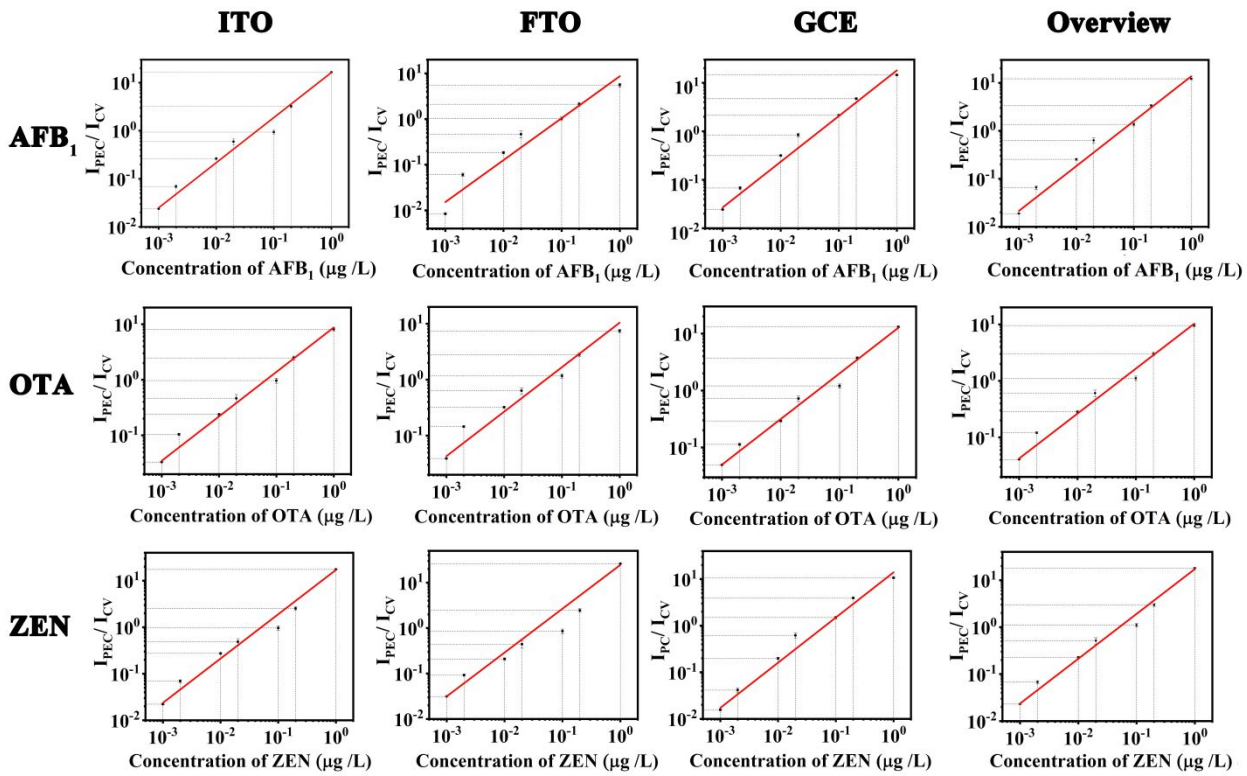

Fig. S3 The corresponding ratio of I_{PEC} and I_{CV} and the total response on ITO, FTO, GCE at different concentrations of AFB_1, OTA and ZEN from 0.001 to 1 μg/L.

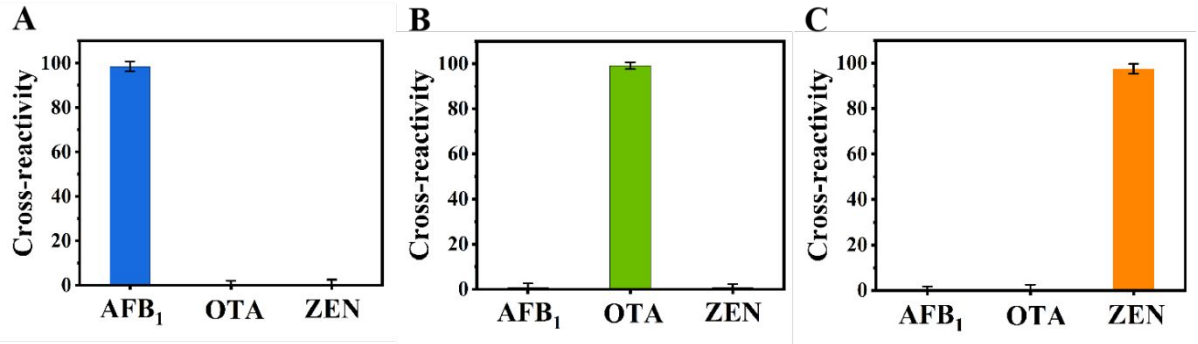

Fig. S4 Cross-reactivity (CR) of immunosensors. The electrode was modified with AFB_1 antigen (A), OTA antigen (B) and ZEN antigen (C).

Table S3 ESI-MS/MS parameters of AFB_1, OTA and ZEN.

Compound	Precursor ion (m/z)	Product ions (m/z)	CD (eV)
AFB_1	313.2 $[M+H]^+$	240.8/285.0	49.9/30.3
OTA	404.1 $[M+H]^+$	239.1/358.0	30.9/18.8
ZEN	317.2 $[M-H]^-$	130.0/174.9	-41.1/-31.2

Table S4 The concentration of OTA, AFB_1 and ZEN in milet and maize by HPLC-MS/MS and this method.

Analyte	Sample	HPLC-MS/MS (μg/kg)	This Work (μg/kg)
AFB_1	Millet	4.2	3.8
	Maize	6.1	6.0
OTA	Millet	60.1	58.2
	Maize	82.0	76.5
ZEN	Millet	0.0	0.0
	Maize	6.2	6.1

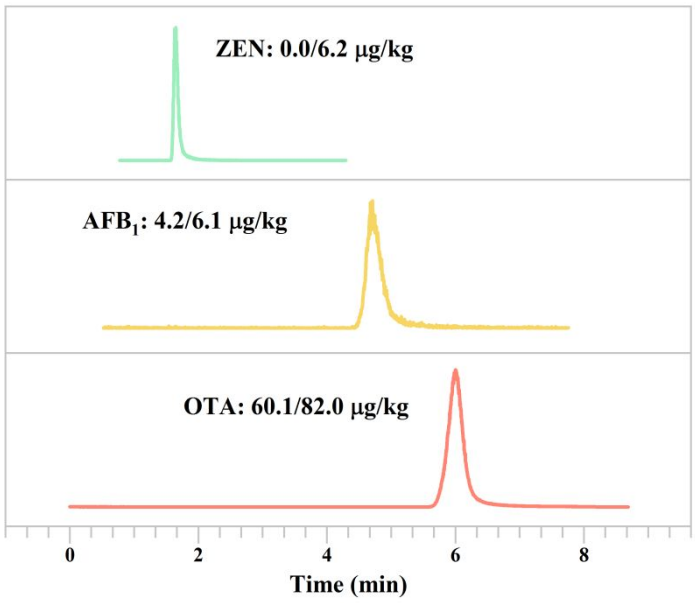

Fig. S5 HPLC-MS/MS chromatograms of ZEN, AFB_1 and OTA.

Table S5 Comparison of analytical performances of different sensors.

Analyte	Measurement	Linear Range (ng/mL)	LOD (pg/mL)	Ref
AFB_1	Immunomagnetic	0.0183-17.9	5.79	2
AFB_1	Electrochemical sensor	0.001-100	6.9	3
AFB_1	This work	0.001-1	0.17	
OTA	Photoelectrochemical immunosensor	0.005-750	1.7	4
OTA	Colorimetric aptasensor	0.05-2	23	5
OTA	This work	0.001-1	0.59	
ZEN	Fluoroimmunoassay	0.038-0.977	12	6
ZEN	Capacitive immunosensor	0.05-5	1.9	7
ZEN	This work	0.001-1	0.6	

References

[1] Lu, F.; Wang, J.; Tao, C.; Zhu, J. J. Highly monodisperse beta-cyclodextrin-covellite nanoparticles for efficient photothermal and chemotherapy. *Nanoscale Horiz.* **2018**, *3*, 538-544

[2] Li, M.; Zhang, Y.; Zhao, R. Immunomagnetic bead-based biotin-streptavidin system for highly efficient detection of aflatoxin B_1 in agricultural products. *RSC Adv.* **2018**, *8*, 26029-26035.

[3] Althagafi, I. I.; Ahmed, S. A.; El-Said, W. A. Fabrication of gold/graphene nanostructures modified ITO electrode as highly sensitive electrochemical detection of aflatoxin B_1. *Plod One* **2019**, *14*, e210652.

[4] Feng, J.; Li, Y.; Gao, Z. Visible-light driven label-free photoelectrochemical immunosensor based on TiO_2/S-$BiVO_4$@Ag_2S nanocomposites for sensitive detection OTA. *Biosens. Bioelectron.* **2018**, *99*, 14-20.

[5] Lin, C.; Zheng, H.; Sun, M. Highly sensitive colorimetric aptasensor for ochratoxin A detection based on enzyme-encapsulated liposome. *Anal. Chim. Acta* **2018**, *1002*, 90-96.

[6] Zhang, F.; Liu, B.; Sheng, W.; Fluoroimmunoassays for the detection of zearalenone in maize using CdTe/CdS/ZnS quantum dots. *Food Chem.* **2018**, *255*, 421-428.

[7] Foubert, A.; Beloglazova N, V.; Hedström, M. Antibody immobilization strategy for the development of a capacitive immunosensor detecting zearalenone. *Talanta* **2019**, *191*, 202-208.