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Development of a simple and convenient cell-based electrochemical biosensor for evaluating the individual and combined toxicity of DON, ZEN, and AFB_1

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ABSTRACT

A simple and convenient cell-based electrochemical biosensor was developed to assess the individual and combined toxicity of deoxynivalenol (DON), zearalenone (ZEN), and Aflatoxin B_1 (AFB_1) on Hep G2 cells. The sensor was modified in succession with AuNPs (gold nanoparticles), cysteamine, and laminin. The cells interacting with laminin formed tight cell-to-electrode contacts, and collagen was used to maintain cell adhesion and viability. Electrochemical impedance spectroscopy (EIS) was developed to evaluate mycotoxin toxicity. Experimental results show that DON, ZEN, and AFB_1 caused a significant decrease in cell viability in a dose dependent manner. The EIS value decreased with concentrations of DON, ZEN, and AFB_1 in the range of 0.01–20, 0.1–50, and 0.1–3.5 μg/mL, and IC_{50} obtained using the developed method was 48.5, 59.0, and 3.10 μg/mL, respectively. A synergistic effect was observed between DON and ZEN, an additive effect was observed between DON and AFB_1, and an antagonism effect was found in the binary mixtures of ZEN and AFB_1 and ternary mixtures. These results were confirmed via CCK-8 assay. Utilizing SEM, we found that cells treated with mycotoxins caused significant changes in cell morphology, thus lessening cell adsorption and impedance reduction. Biological assay indicated that EIS patterns correlated with $[Ca^{2+}]_i$ concentrations and apoptosis and necrotic cells ratios, thus effecting electrochemical signals. This method is simpler, more convenient, sensitive, and has a quicker response rate than most conventional cytotoxicity evaluation methods.

1. Introduction

The term mycotoxin designates a chemically diverse group of secondary fungal metabolites (Binder et al., 2007), mainly produced by the *Aspergillus*, *Penicillium*, or *Fusarium genus* (Streit et al., 2012). Contamination is largely dependent on environmental factors and may occur in the field or during improper storage (Marroquin-Cardona et al., 2014). These mycotoxins will cause a toxic response called mycotoxicosis when ingested by humans or animals above a certain concentration (Wen et al., 2016). Among these, AFB_1 is the most toxic and carcinogenic among the known mycotoxins and has been shown to be hepatocarcinogenic, mutagenic, and genotoxic (Lei et al., 2013a). ZEN is a non-steroidal, estrogenic mycotoxin, and is one of the most widely distributed fusarium mycotoxins, as it is encountered at high incidence in many crops for human and animal consumption. DON, also known as vomitoxin, is a member of the trichothecenes family commonly detected in cereals and grains. The main risks DON poses to humans primarily involves the intestinal, central nervous, and immune systems.

Most toxin producing fungi are capable of producing multiple mycotoxins concurrently, and food and feed can be contaminated with various fungal species at the same time. Humans and animals are likely to be exposed to a range of mycotoxins simultaneously (Streit et al., 2012). This is particularly important regarding DON, ZEN, and AFB_1, which are so ubiquitous and co-occur in a range of commodities (Li et al., 2014; Wu et al., 2016). It is therefore necessary to assess the concomitant action of these three mycotoxins. In vitro assays on cells have the advantage of minimizing animal use, allowing for the testing of a wide range of chemicals and concentrations. Considering that oral exposure is the most common mycotoxin contamination route and that the liver is the initial site of metabolism in humans. We selected the human hepatoma Hep G2 cells as the in vitro experimental system.

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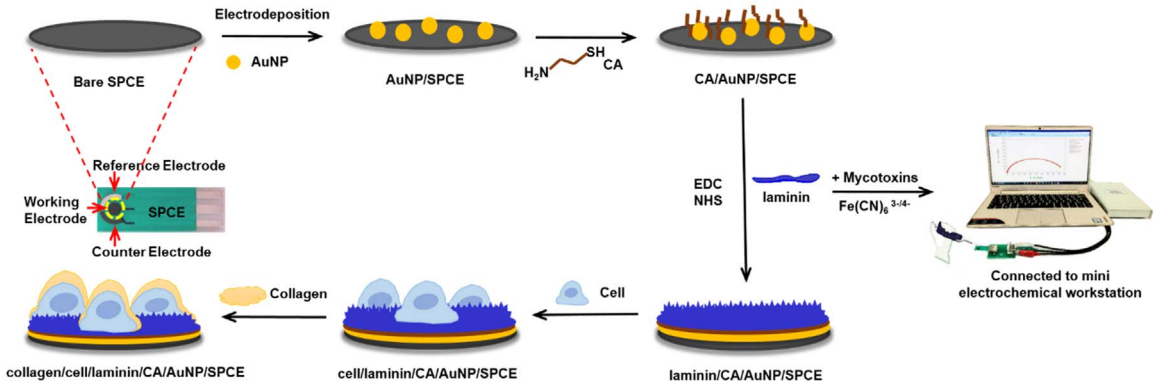

Scheme 1. Schematic representation of the route for the preparation of the cell-based electrochemical sensor.

Numerous bioassays have been developed to evaluate cytotoxicity, but these traditional evaluation methods are time consuming, labor intensive, and require complex processes, including cell lysis or fixation at prescheduled time points. There is thus a strong demand for label-free detection methods for cell-based assays (Ceriotti et al., 2007). In recent years, new cytotoxicity tests based on the electrochemical behavior of living cells have been developed, among which EIS has emerged as one of the most interesting label-free technology, as an powerful alternative for monitoring interfacial property changes at the electrode surface (Xing et al., 2005). It has also been successfully used to monitor adhesion, cell growth and differentiation, morphological changes during apoptosis, taste sensor, and cytotoxicity of several drugs including mycotoxins (Ceriotti et al., 2007; Curtis et al., 2009; Xu et al., 2015).

In this study, we used screen-printed carbon electrodes (SPCEs) rather than traditional electrodes. The SPCEs can effectively develop portable biosensors to be used in electroanalysis because they are inexpensive and easy to mass produce (Seenivasan et al., 2015). Cell immobilization on the electrode surface is essential for biosensor design. Biocompatible coating material is a convenient way to improve the efficiency of this cell immobilization. Cells are thus often coupled with a sensor surface using different surface modifications, including peptides, extracellular matrices (ECM), and self-assembled monolayers (SAM) (Robertus et al., 2010). ECM components, such as polylysine, laminin, fibronectin, and collagen can provide better adherence and cell spreading on the biosensors (Asphahani and Zhang, 2007). In this study, we used cells interacting with laminin (adhesion proteins) anchored via chemisorbed SAM layers form tight cell-to-electrode and tight cell-to-cell contacts that monitor cell electrical impedance (Curtis et al., 2009; Slaughter and Hobson, 2009). Type I collagen, which has excellent biocompatibility, was used to encapsulate the Hep G2 cells and provide a three-dimensional capsule structure for cell proliferation and viability (Jiang et al., 2013).

We present a novel and simple method for designing a cell-based impedance sensor to evaluate the individual and combined toxicity of DON, ZEN, and AFB_1. Differing electrochemical responses caused by mycotoxin stimulation on the Hep G2 cells are discussed below. We then compared the EIS and CCK-8 assay methods, calcium concentration changes, and cell apoptosis, and found that the EIS signal corresponded with calcium concentration changes and cell apoptosis. The results show that the proposed method is simple, convenient, and exhibits a quick response rate. This study provides a promising alternative to conventional mycotoxin cytotoxicity evaluation methods.

2. Experimental procedures

2.1. Materials and apparatus

Chloroauric acid and Cysteamine (CA) were purchased from Sinnpharm Chemical Reagent Co., Ltd (Shanghai, China). Laminin, N-(3-dimethylaminopropy)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), DON, ZEN, and AFB_1 were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Type I collagen (rattail, collagen-I) was purchased from YESEN Biological Technology Co., Ltd (Shanghai, China). Liver hepatocellular cells (Hep G2) were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All solutions were prepared with ultrapure water and all reagents were of analytical grade.

SPCEs and corresponding linking devices were obtained from Shanghai Molybdenum Electronic Technology Co., Ltd. Electrochemical experiments were performed with the CHI660e electrochemical workstation (Chenhua, Shanghai, China) on an SPCE system. Morphologies of the modified electrode were characterized under a scanning electron microscope (model Hitachi SU8000 and S-4800, Hitachi Co., Ltd., Tokyo, Japan). The CCK-8 and fluorescence intensity assays were measured using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunny vale, CA, USA) and FACS (Becton-Dickson, San Jose, CA, USA).

2.2. Cell culture

Hep G2 cells were cultured in a flask in a high-glucose DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin at 37 ℃ in a humidified atmosphere containing 5% CO_2. The cells reached a concentration of 5.0×10^6 cells/mL at a growth retardation stage of 3 days, after which they were used for experiments.

2.3. Laminin-modified SPCE preparation and cell immobilization

The electrochemical biosensor stepwise fabrication is shown in Scheme 1. Disposable triple-electrode SPCEs were used in this study. Bare SPCEs were scanned from −0.2 V to + 0.6 V in 2.5 mM of $Fe(CN)_6^{3-/4-}$ 10 times cyclic voltammetry (CV) to remove electrode surface oxides before working electrode modification. We then used electrodeposition to deposit AuNPs on the SPCE in a 0.5 M H_2SO_4 solution containing 1 mM of $HAuCl_4$ under a constant potential of −0.25 V for an optimal time of 120 s to improve electron transfer efficiency. The electrode was then washed in ultrapure water. Immediately after cleaning, the AuNP/SPCE was placed into an 18 mM CA aqueous solution at room temperature in darkness for 4 h to form a modified Au-CA electrode, then removed from the CA solution, rinsed multiple times with water to eliminate physically adsorbed CA, and dried with N_2. The modified CA SAM electrode was then treated with covalent immobilization of laminin to the SAM monolayers. The electrode was then incubated under hetero-bifunctional coupling conditions with 10 μL of 100 μg/mL laminin via the EDC and NHS and incubated for 2 h. This resulted in an NHS-activated laminin site, and improved laminin coupling efficiency to the CA/

AuNP/SPCE electrodes. After this, 10 μL of Hep G2 cells suspension at a concentration of 10^6 cells/mL was dropped onto the laminin/CA/AuNP/SPCE surface, and incubated at 37 ℃ for 12 h before being immersed in a PBS buffer to remove uncaptured cells. Finally, 10 μL collagen was dropped onto the surface to maintain cell adhesion and viability.

2.4. Electrochemical measurements

Electrochemical experiments were performed on a CHI660e electrochemical workstation with a disposable SPCEs (3 mm diameter carbon working electrode, carbon counter electrode, and silver/silver chloride reference electrode). The electrochemical behavior of Hep G2 cells exposed to various mycotoxin concentrations in DMEM was studied via EIS carried out in the presence of 2.5 mM $Fe(CN)_6^{3-/4-}$ and 1.0 M KCl. Cells not exposed to mycotoxins served as the control. The impedance spectra were recorded within a frequency range of 1–10^5 Hz. The EIS dates were captured in ZView software (Gu et al., 2015). All electrochemical experiments were carried out at room temperature. Mycotoxin cytotoxicity was calculated as follows (Chen et al., 2005; Gu et al., 2015):

$$\text{Cytotoxicity}(\%) = 100[(R_{collagen} - R_{mycotoxin})/(R_{cell} - R_{laminin})] \quad (1)$$

Where $R_{laminin}$ is laminin modified electrode impedance, R_{cell} is immobilized cell impedance on electrode, $R_{collagen}$ is collagen packed cell impedance, and $R_{mycotoxin}$ is collagen packed cell impedance treated with certain mycotoxin concentrations. Other experimental parameters are as follows: Cyclic voltammetry (CV) had a scan range of −0.2–0.6 V, and scan rate of 0.1 V/s; Differential pulse voltammetry (DPV) had a scan range of −0.2–0.6 V, pulse amplitude of 0.1 V.

2.5. Statistical analysis

Data are expressed as mean ±S.D. Statistical analysis was carried out with SPSS 17.0. Differences were determined utilizing the unpaired Student's *t*-test and set at $p < 0.05$. Individual mycotoxin dose-response relationships were biometrically modeled using the Median-Effect Equation (Chou and Talalay, 1984):

$$\left(\frac{f_a}{f_u}\right) = \left(\frac{D}{D_m}\right)^m \quad (2)$$

where D is concentration, D_m is concentration for the 50% effect, f_a is the fraction affected by concentration D, f_u is the unaffected fraction ($f_a = 1 - f_u$), and m is the sigmoid coefficient of the dose-response curve: where $m = 1$, $m > 1$, and $m < 1$ indicate hyperbolic, sigmoidal, and negative sigmoidal dose-response curves, respectively.

Mycotoxin interactions were analyzed utilizing the isobologram and combination index (CI) methods derived from Chou and Talalay's median effect principle (Chou and Talalay, 1984). This index was then calculated according to (Chou, 2011):

$$^nCI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} \quad (3)$$

where $^n(CI)_x$ is combination index for n toxins at $x\%$ inhibition, $(D)_j$ is doses of n toxins that exert $x\%$ inhibition in combination, $(D_x)_j$ is doses of each n toxins alone exerting $x\%$ inhibition; CI = 0. 9 − 1. 1, CI<0.9, CI>1. 1 indicates an additive effect, a synergism, and an antagonism, respectively, regardless of the mechanisms or drug units. The effect parameters of single and combined drugs on inhibition of HepG2 cells for 24 h were calculated in CompuSyn software.

3. Results and discussion

3.1. Modified screen-printed carbon electrode characterization

Scheme 1 illustrates the entire electrode modification and SPCE procedure with the corresponding connecting device. Individual SPCE shape was rectangular at 10 mm × 33 mm. Electroconductive silver paste was printed on polyethylene terephthalate (PET) substrate as the first layer followed by a second layer of elelctroconductive carbon paste. An insulation layer was then used to cover the exposed work area. We modified the SPCE with AuNPs to further enhance SPCE sensitivity. The AuNPs were uniformly deposited on clean SPCE surface, as shown in Fig. S1 and Fig. S3B. The AuNPs distribution on the electrode surface increased the surface area and improved electrochemical sensitivity. The amount of AuNPs on the electrode was determined by the deposition time, which was optimized by measuring DPV (Fig. S2). A deposition of 120 s was selected according to optimization results to modify electrode. The CAs containing a thiol residue on the terminus were then self-assembled on the AuNP/SPCE via an Au-S covalent bond, and the amino group on the other terminus was used to react with the laminin carboxyl group. Laminin was then covalently coupled to the electrode. Cells interacting with laminin anchored via chemisorbed SAM layers formed tight cell-to-electrode and tight cell-to-cell contacts, displaying a high affinity between laminin receptors and laminin. Finally, collagen was used to maintain cell adhesion and viability. The SPCE surface morphology after all electrode fabrication steps was characterized using SEM imaging (Fig. S3).

3.2. Electrochemical characteristics of Hep G2 cells on a modified electrode

CV and DPV were used to characterize both the biosensor process and cell adsorption on the sensor surface. As shown in Fig. 1A, the current increased significantly when the bare SPCE was modified with AuNPs (curve b) due to its excellent conductivity and increased working electrode's effective area. Laminin and CA modification on the electrode surface resulted in a decrease in the $Fe(CN)_6^{3-/4-}$ peak current. This phenomenon is attributed to CA molecules and laminin dampening the redox reaction. We observed a decrease in peak current and a drastic increase in peak separation when Hep G2 cells and collagen adhered to the sensor surface. Peak current and separation were caused by electron transfer blockage in the the cell membrane and collagen insulation, leading to increased electrode resistance. Some similar signal changes are shown in Fig. 1B. The EIS of modified electrode and cell adhesion are shown in Fig. 1C. The EIS spectra of $Fe(CN)_6^{3-/4-}$ probe showed that different modified electrodes possessed different electron-transfer resistances (R_{et}). With bare SPCE, resistance was 13,410 Ω, and the semicircle decreased dramatically when AuNPs were assembled on the bare SPCE. The R_{et} value was approximately 700 Ω. The CA and laminin were then modified on the AuNP/SPCE surface, and the R_{et} value increased to 2252 Ω and 6174 Ω, respectively. Hep G2 cells attached to the laminin/CA/AuNP/SPCE cells hampered the redox probe and thus increased R_{et}. After collagen encapsulated the cells, the R_{et} increased to 13,030 Ω, indicating that electron transfer became more difficult.

These EIS results are similar to the changes in CV and DPV signals. Compared to other electrochemical methods, EIS is a noninvasive and highly sensitive method. We then chose EIS to monitor the proliferation and apoptosis of Hep G2 cells on the electrode surface. As shown in Fig. S4, cells numbering 1×10^6 cell/mL and an adsorption time of 10 h were selected for analysis.

3.3. Electrochemical characteristics of laminin effects on cell immobilization

We used CA/AuNP/SPCE-modified electrode as a control to

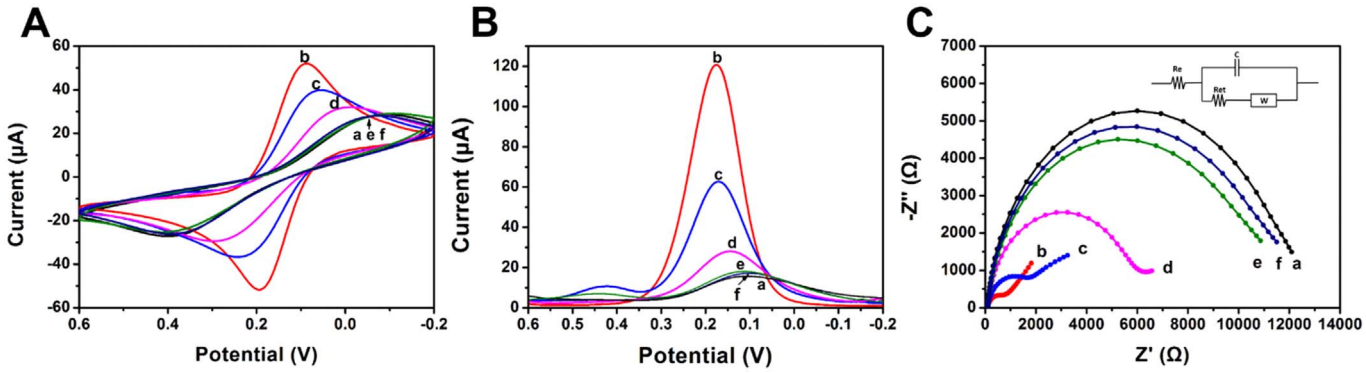

Fig. 1. Electrochemical characterization of the modified screen printed carbon electrode. (A) Cyclic voltammograms of (a) bare SPCE, (b) AuNP/SPCE, (c) CA/AuNP/SPCE, (d) laminin/CA/AuNP/SPCE, (e) cell/laminin/CA/AuNP/SPCE, (f) collagen/cell/laminin/CA/AuNP/SPCE. (B) Differential pulse voltammetry of (a) bare SPCE, (b) AuNP/SPCE, (c) CA/AuNP/SPCE, (d) laminin/CA/AuNP/SPCE, (e) cell/laminin/CA/AuNP/SPCE, (f) collagen/cell/laminin/CA/AuNP/SPCE. (C) Nyquist diagrams of electrochemical impedance spectra of (a) bare SPCE, (b) AuNP/SPCE, (c) CA/AuNP/SPCE, (d) laminin/CA/AuNP/SPCE, (e) cell/laminin/CA/AuNP/SPCE, (f) collagen/cell/laminin/CA/AuNP/SPCE in $Fe(CN)_6^{3-/4-}$, scan rate: 100 mV S^{-1}. The insets is the equivalent circuit model, to which all of the experimental impedance curves were fitted.

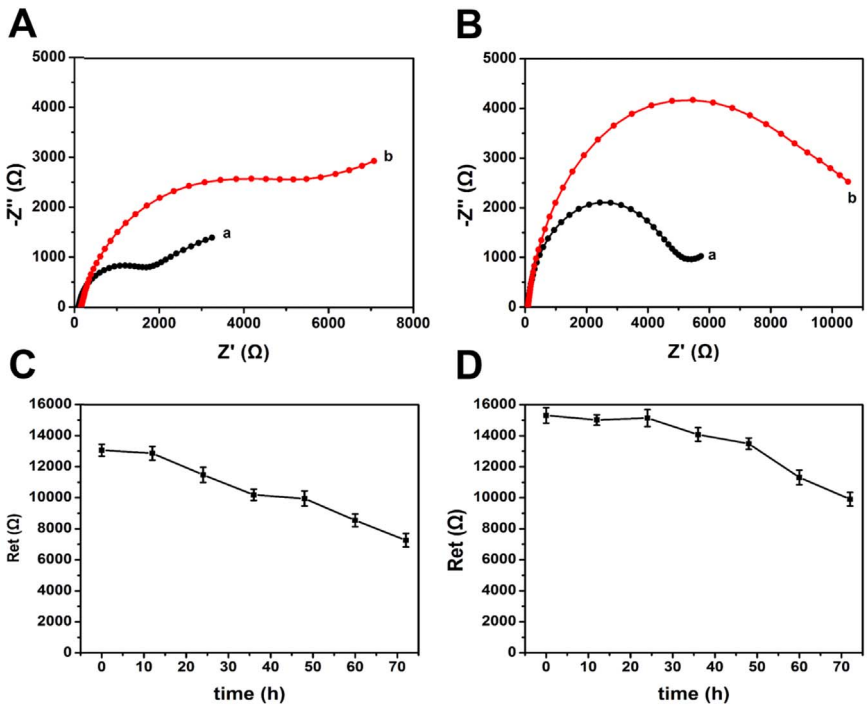

Fig. 2. Electrochemical impedance spectroscopy (EIS) of (A): (a) CA/AuNP/SPCE, (b) cell/CA/AuNP/SPCE and (B): (a) laminin/CA/AuNP/SPCE, (b) cell/laminin/CA/AuNP/SPCE. Electrochemical characteristics of the effect of collagen, as culture time increased, Nyquist diagrams of (C) the cell/laminin/CA/AuNP/SPCE modified electrode without collagen and (D) the modified electrode with collagen. (a) 0 h, (b) 12 h, (c) 24 h, (d) 36 h, (e) 48 h, (f) 60 h, (g) 72 h.

investigate the effects of laminin on cell immobilization on the electrode surface. The other electrode used was a laminin/CA/AuNP/SPCE modified electrode. We added 10 μL of Hep G2 cell suspension at a concentration of 1×10^6 cell/mL to both electrode surfaces, where they were incubated at 37 °C for 10 h. The non-adherent cells were then removed with a PBS buffer (pH 7.4). As shown in Fig. 2, the R_{et} of the CA/AuNP/SPCE-modified electrode increased from 2250 Ω to 9905 Ω (Fig. 2A), while the R_{et} of the laminin/CA/AuNP/SPCE-modified electrode increased from 5670 Ω to 13,490 Ω (Fig. 2B). The results show that laminin promoted cell immobilization on the electrode surface, thus leading to an increase in R_{et}. These cells carried transmembrane proteins called integrin on their surface. Integrin receptors link cells to laminin and mediate signaling between cells and electrodes (Robertus et al., 2010). Laminin can bind to these cell receptors to immobilize the cell on the electrode surface. Thus, cells interacting with laminin can form tight cell-to-electrode and tight cell-to-cell contacts that monitor cell electrical impedance.

3.4. Electrochemical characteristics of collagen to maintain cell adhesion and viability

We used the cell/laminin/CA/AuNP/SPCE without collagen as control to investigate the effects of collagen on maintaining cell adhesion and viability. We found that the R_{et} value of the control decreased as time increased from 0 h to 72 h. However, the R_{et} value with collagen decreased slightly, remaining constant for the first 24 h. The modified electrode impedance is related to cell number and viability, indicating that the modified electrode without collagen shows decreasing cell viability. This may be due to some cells dropping off the electrode or to a nutrient shortage after a long period of decrease in cell

viability. These results suggest that collagen can supply cells with nutrition, thus maintaining adhesion and viability.

3.5. Hep G2 cell sensor evaluate the toxicity of DON, ZEN, AFB_1 and their combinations

The electron-transfer resistance was related to immobilized cell count on the modified electrode and to the morphology and apoptosis of the adhered cells (DePaola et al., 2001; Wang et al., 2016). Thus, in addition to the detection of cell immobilization, this electrochemistry method can be used to monitor both the morphological changes and cell apoptosis on electrode surface in real time. In this study, the modified electrodes were exposed to different concentrations DON, ZEN, and AFB_1 for 24 h to analyze the individual mycotoxin toxicity. Typical electrochemical impedance spectroscopy results are presented in Nyquist diagrams (Fig. S5), in which a distinct change in Hep G2 cell impedance value was observed. As DON concentration increased from 0.01 to 50 μg/mL, the inhibition rate of Hep G2 cells significantly increased from 5.1% to 53.91%, as measured by EIS. The inhibition rate increased from 0.1% to 64.14% after ZEN exposure increased from 0.1 to 50 μg/mL, and the inhibition rate increased from 1.92% to 89.9% after AFB_1 exposure increased from 0.1 to 3.5 μg/mL. The developed electrochemical toxicity evaluation method showed increasing mycotoxin cytotoxicity in a dose-dependent manner.

These results indicate that DON, ZEN, and AFB_1 all reduced Hep G2 cell viability as expected. We found AFB_1 to be more effective at reducing cell viability, while ZEN exhibited weak cytotoxicity. The mycotoxin concentration corresponding to half maximum cytotoxicity was defined as IC_{50}, which for DON, ZEN, and AFB_1 was 48.5, 59.0, and 3.10 μg/mL, respectively, as obtained by EIS. The CCK-8 assay was performed to assess cell viability to compare with the electrochemical method results. Our conclusion was confirmed by the CCK-8 assay, as shown in Fig. 3. We found that EIS was more sensitive at low concentrations compared with the CCK-8 assay. The IC_{50} of DON, ZEN, and AFB_1 were 40.38, 40.38, and 3.06 μg/mL, respectively. These values obtained using the CCK-8 assay were very close to those obtained using the proposed electrochemical method. The electrochemical method was simple and convenient at evaluating cell viability via EIS, while conventional methods are time-consuming and complex.

Five concentrations of DON (0.01, 0.5, 1, 10 and 20 μg/mL), ZEN (5, 10, 20, 40 and 50 μg/mL), and AFB_1 (2.5, 2.8, 2.9, 3 and 3.1 μg/mL) below IC_{50} were used to assess the combined toxicity of DON, ZEN and AFB_1. After exposure to DON + ZEN (at 0.01 + 5, 0.5 + 10, 1 + 20, 10 + 40 and 20 + 50 μg/mL), DON + AFB_1 (at 0.01 + 2.5, 0.5 + 2.8, 1 + 2.9, 10 + 3, and 20 + 3.1 μg/mL), ZEN + AFB_1 (at 5 + 2.5, 0 + 2.8, 20 + 2.9, 40 + 3, and 50 + 3.1 μg/mL), and DON + ZEN + AFB_1 (at 0.01 + 5 + 2.5, 0.5 + 10 + 2.8, 1 + 20 + 2.9, 10 + 40 + 3, and 20 + 50 + 3.1 μg/mL) for 24 h, cytotoxic interactions were assayed utilizing the isobolo-

Table 1.
Combination index (CI) values for the different mycotoxin combinations using the proposed electrochemical method.

Mycotoxin combination, μg/mL			Inhibition of viability/100, *fa*	CI	Indication
DON	ZEN	AFB_1			
0.01	5		0.092	0.86262	Synergism
0.5	15		0.297	0.50479	Synergism
1	20		0.385	0.59996	Synergism
10	40		0.5076	0.84755	Synergism
20	50		0.7151	0.39734	Synergism
0.01		2.5	0.002	27955.3	Antagonism
0.5		2.8	0.493	0.91637	Additive effect
1		2.9	0.445	0.99064	Additive effect
10		3	0.5095	1.14902	Antagonism
20		3.1	0.6589	1.01069	Additive effect
	5	2.5	0.092	1.60833	Antagonism
	15	2.8	0.182	1.65674	Antagonism
	20	2.9	0.464	1.33089	Antagonism
	40	3	0.6892	1.24635	Antagonism
	50	3.1	0.9722	0.81037	Synergism
0.01	5	2.5	0.111	1.591	Antagonism
0.5	15	2.8	0.369	1.26457	Antagonism
1	20	2.9	0.385	1.56749	Antagonism
10	40	3	0.445	2.21028	Antagonism
20	50	3.1	0.5076	2.19842	Antagonism

CI < 0.9, CI = 0.9–1.1, and CI > 1.1 indicate synergism, additive effect, and antagonism, respectively. Computer software CompuSyn was used for calculation and simulation.

gram method, which provides CI values as a quantitative measure of the three mycotoxin interaction degrees. The results are shown in Table 1, and indicate that Hep G2 cell susceptibility towards mycotoxins differs depending on the combinations assayed. All mixtures showed reduced cell viability compared to the control. Binary and tertiary combinations showed a dose dependent effect. A synergistic effect was observed between DON and ZEN, and an additive effect between DON and AFB_1. By contrast, binary mixtures of ZEN and AFB_1 and ternary mixtures of DON, ZEN, and AFB_1 showed an antagonism effect. Evaluation results that were obtained by the proposed electrochemical method and the CCK-8 assay were very similar (Table S1).

In general, the electrochemical cytotoxicity evaluation results agreed with the results of CCK-8 assay, the slight different might be attributed to the different culture conditions and assay procedures. The outcomes of mixtures are similar to those observed by Gu et al. (2015), Lei et al. (2013b), while divergences occurring in the ternary mixture showed synergistic effects observed by Sun et al. (2015). These divergences maybe related to the different type of cells used in these different studies, as mycotoxins have varying effects on various organs

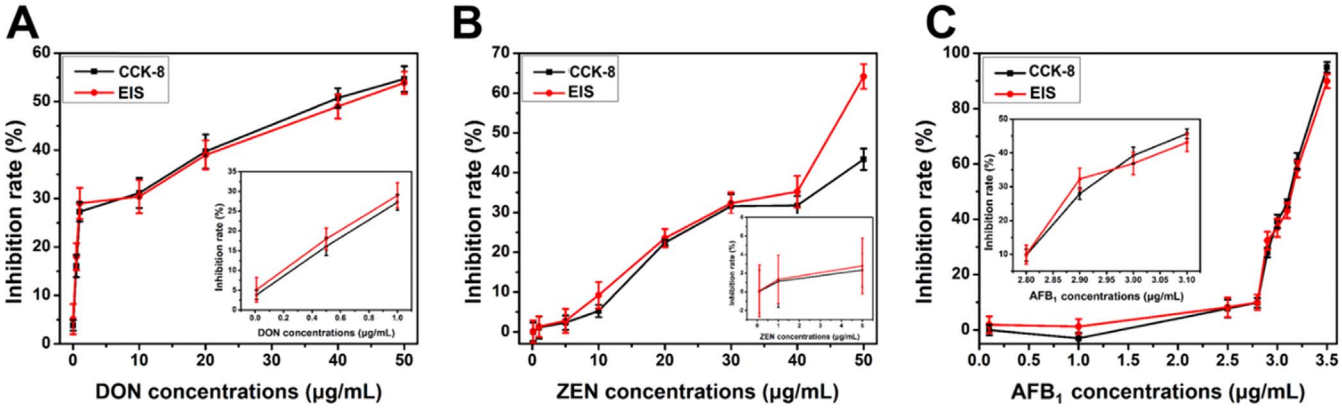

Fig. 3. Cytotoxicity curves for 24 h exposure of the Hep G2 cells on DON, ZEN and AFB_1 obtained by obtained by using CCK-8 assay and the proposed electrochemical method. (A) DON: 0.01 ,0.5, 1, 10, 20, 40, and 50 μg/mL. (B) ZEN: 0.1, 1, 5, 10, 20, 30, 40, and 50 μg/mL. (C) AFB_1:0.1, 1, 2.5, 2.8, 2.9, 3, 3.1, 3.2, and 3.5 μg/mL.

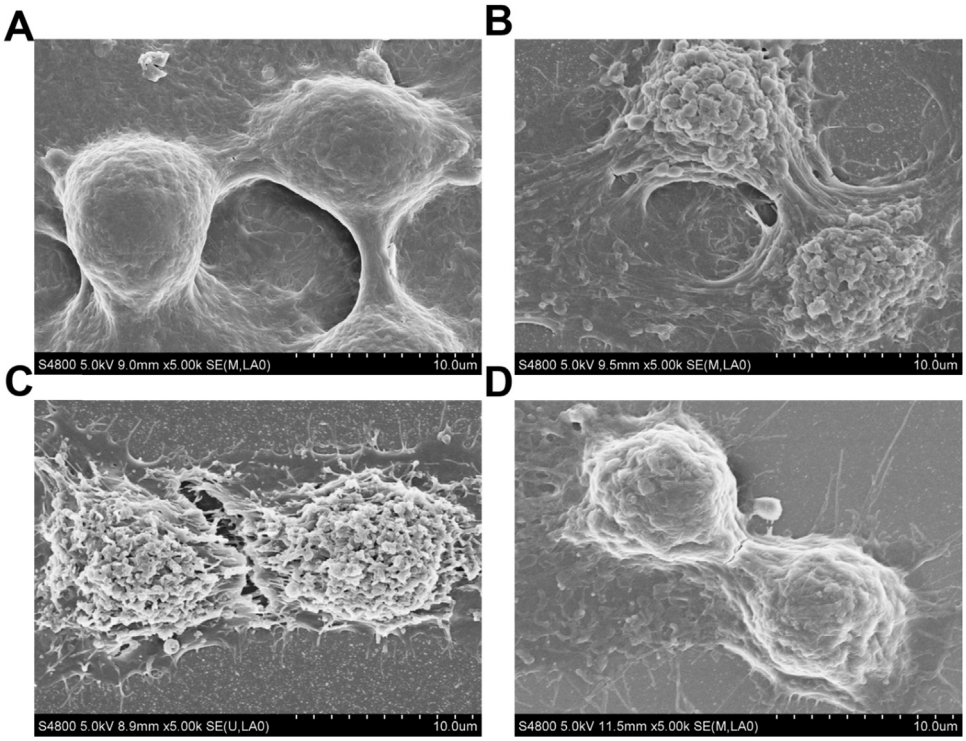

Fig. 4. The morphology of cells treated with different mycotoxin for 24 h. (A) Control: cells non-treated with mycotoxin. (B) Cells treated with 1 μg/mL DON. (C) Cells treated with 20 μg/mL ZEN. (D) Cells treated with 2.9 μg/mL AFB_1. In this case, the inhibition rate was about 30%.

with different concentrations, exposure durations, and experimental parameters. Our method is a simple, rapid, and cost-efficient approach to testing combinations of mycotoxins and to obtaining a first glimpse of their toxic potential.

3.6. *Effects of DON, ZEN and AFB_1 on Hep G2 cells*

Mycotoxins induced a significant impedance decrease, as shown by EIS after exposure to different mycotoxin doses for 24 h. This method was more sensitive compared with the CCK-8 assay. Non-treated Hep G2 cells retained their common shape and were integrated, widely spread, and adhered tightly to the surface, emit pseudopods on the surface of cell plasma membrane, as shown in the SEM image in Fig. 4. However, Hep G2 cells treated with mycotoxins underwent significant changes in cell morphology (Fig. 4B-D). The cell surface became rough and perforated, there was cytoplasm loss, plasma membrane blebbing, and apoptotic cells detached from the substrate and neighboring cells. Furthermore, we found cell numbers decreased and some became complete lyses. This is a possible reason the impedance value decreased as mycotoxins concentration increased, while the diameter of the semi-circle on the Nyquist diagram shrinks.

We also investigated the effects of DON, ZEN, and AFB_1 on calcium ion production and cell apoptosis in Hep G2 cells to explore inherent cell change. The operation is detailed in the supporting information. Calcium ion production did not change with DON treatment, as shown in Fig. S7A, but cells with ZEN and AFB_1 for 24 h induced an increase in intracellular Ca^{2+} levels (Fig. S7B and C). The inhibition rate by EIS and relative fluorescence intensity on calcium reflected a nearly linear relationship, indicating that calcium mobility might affect electron transfer and improve electrical signal. The percentage of necrotic, late apoptotic, early apoptotic, and viability is shown in Fig. S6. Marked changes in cell profiles after mycotoxin treatment revealed a significant decrease in viable cells and a slight increase in early apoptotic cells after 24 h treatment with either DON, ZEN, or AFB_1. In addition, a sharp increase in necrotic cells were observed when cells were treated with individual high-dose mycotoxin. Furthermore, the inhibition rate by EIS and the apoptosis and necrotic cell percentages have similar dose-response trends.

4. Conclusion

Sensors that evaluate cellular cytotoxic responses are well-suited for a broad range of sensing applications, including the detection of unknown agents. The use of mammalian cells as biosensors can provide responses relevant to human physiology. Many toxicity sensors have been proposed, however, there are few studies adopting electrochemical sensors to evaluate combined mycotoxin toxicity. In this study, a simple, convenient, and sensitive Hep G2 cell-based electrochemical biosensor was developed by immobilizing living cells on a modified-electrode surface to evaluate the individual and combined toxicity of DON, ZEN, and AFB_1. The electrochemical mycotoxin sensitivity test offers potential advantages over conventional assays. Our results show that DON, ZEN, and AFB_1 caused a marked decrease in the cell viability in a dose-dependence manner. Electrochemical impedance spectroscopy value decreased with the concentration of DON, ZEN, and AFB_1 in range of 0.01–20, 0.1–50, and 0.1–3.5 μg/mL, respectively, and the IC_{50} for DON, ZEN, and AFB_1 as obtained by the developed electrochemical method was 48.5 μg/mL, 59.0 μg/mL and 3.10 μg/mL, respectively. A synergistic effect was observed between DON and ZEN, an additive effect appeared between DON and AFB_1, and binary mixtures of ZEN and AFB_1 revealed an antagonism effect. Ternary mixtures of DON, ZEN, and AFB_1 also showed an antagonism effect. These results were confirmed by CCK-8 assay. With EIS measurements, the cell-based electrochemical biosensor can be used to evaluate mycotoxin toxicity in Hep G2 cells when morphological changes are generated on cell surface. The EIS was correlated in cells with calcium ion concentration and the apoptosis and necrotic cell percentages, suggesting that intracellular calcium ion production and cell apoptotic took place in cells after incubation with mycotoxins, thus effecting the electrochemical signals. The electrochemical approach is highly sensitive, in real-time, simple, and convenient, and could be developed as a convenient means to study mycotoxin toxicity. Further research is

necessary to verify mycotoxin combinations and long-term modified electrode storage. Our study is the first to present a novel method for designing a cell-based impedance sensor to evaluate the combined toxicity of DON, ZEN, and AFB_1.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2017.06.002.

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