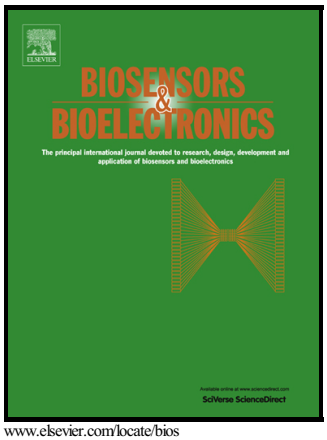

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A novel and simple cell-based electrochemical impedance biosensor for evaluating the combined toxicity of DON and ZEN

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Abstract:

In this study, a novel and simple cell-based electrochemical biosensor was developed to assess the individual and combined toxicity of deoxynivalenol (DON) and zearalenone (ZEN) on BEL-7402 cells. The sensor was fabricated by modification with AuNPs, p-aminothiophenol, and folic acid in succession. The BEL-7402 cells which had a good activity were adhered on the electrode through the high affinity between the folate receptor and folic acid selectivity. We used the collagen to maintain the cell adhesion and viability. Electrochemical impedance spectroscopy (EIS) was developed to evaluate the individual and combined toxicity of DON and ZEN. Our results indicate that DON and ZEN caused a marked decrease in the cell viability in a dose-dependent manner. The value of electrochemical impedance spectroscopy decreased with the concentration of DON and ZEN in range

of 0.1-20, 0.1-50 μg/ml with the detection limit as 0.03, 0.05 μg/ml, respectively, the IC_{50} for DON and ZEN as obtained by the proposed electrochemical method were 7.1 μg/ml and 24.6 μg/ml, respectively, and the combination of two mycotoxins appears to generate an additive response. The electrochemical cytotoxicity evaluation result was confirmed by biological assays. Compared to conventional methods, this electrochemical test is inexpensive, highly sensitive, and fast to respond, with long-term monitoring and real-time measurements. The proposed method provides a new avenue for evaluating the toxicity of mycotoxins.

Keywords: DON, ZEN, combination, toxicity, electrochemistry.

1. Introduction

Mycotoxins are natural contaminants that are produced by filamentous fungi. The presence of these harmful compounds in food and feed has long been recognized as a potential human and animal health hazard (Bennett and Klich 2003). Deoxynivalenol (DON), a representative mycotoxin of the trichothecene B group, is one of the most widespread cereal contaminants worldwide (Krysinska-Traczyk et al. 2007). At a cellular level, the main toxic effect of DON is due to the inhibition of protein and nucleic acid synthesis via binding to the ribosome and by activating cellular kinases that are involved in signal transduction, which consequently results in decreased cell proliferation. Zearalenone (ZEN) is also one of the most frequent fusariotoxins in small grain cereals (Scudamore and Patel 2000). ZEN is a non-steroidal estrogenic mycotoxin that induces genotoxic and carcinogenic disorders

by producing DNA adducts and micronuclei and by causing chromosome aberrations (Ayed-Boussema et al. 2007).

Most studies have evaluated the effect of mycotoxins taken individually. Nevertheless, it is very likely that humans and animals are exposed to a mixture rather than to individual compounds, which is particularly true for DON and ZEN, which can both be produced by Fusarium culmorum and are quite common contaminants that occur jointly in several cereal grains. Mycological surveys carried out on freshly harvested wheat grains from the main production regions in the northern part of Tunisia revealed that grains were heavily contaminated with mycotoxin-producing fungi, and the most recovered genus in terms of frequency was Fusarium, predominately F. culmorum and F. pseudograminearum (Bensassi et al. 2011). Indeed, these Fusarium Head Blight (FHB)-associated pathogens are capable of producing a number of mycotoxins, including DON and ZEN. Thus, chromatographic analyses that were conducted by Bensassi (Bensassi et al. 2010) and Zaied (Zaied et al. 2012) showed the natural occurrence of both DON and ZEN in wheat that was produced under Tunisian agroclimatic conditions with levels that exceeded in several samples DON/ZEN limit that is fixed by the European Commission Reg.1881/2006 intended for human and animal consumption. Thus, it would be interesting to assess the effects of the concomitant action of the two fusarial toxins DON and ZEN.

Considering that oral exposure is the route of contamination by DON and that the liver is the initial site of DON metabolism in human, P450 may play an important role in the oxidation metabolism of DON in this organ. In addition, hepatocytes

constitute a target system for ZEN toxicity, and the liver is a prominent site for metabolism and for intense oxidative processes in the body. In conclusion, we selected BEL-7402 cells as the in vitro experimental system in our study.

Biological assays of cell activity and viability evaluation, such as by measuring the mitochondrial reduction of tetrazolium salts into an insoluble dye (the MTT test), the release of the enzyme lactate dehydrogenase (LDH), and the measurement of reactive oxygen species (ROS), and mitochondrial apoptosis are often used to evaluate toxicity (Thannickal and Fanburg 2000). The MTT assay is a colorimetric assay which also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of potential medicinal agents and toxic materials(Cetin and Bullerman 2005). These methods are complex, costly, and difficult for the long-term monitoring of cells. To simplify the test procedure, reduce costs, and enhance sensitivity, cytotoxicity tests based on the electrochemical behavior of living cells, such as electron transfer at electroactive centers in cells (Li et al. 1999), open circuit potential at the cell/sensor interface (Woolley et al. 2002), and electric cell-substrate impedance (Xiao et al. 2002), have been developed. In contrast, electrochemical sensing to assess toxicity is inexpensive, highly sensitive, selective, fast to respond, small, and capable of long-term monitoring or real-time measurements and of generating reproducible results (Rahman 2011). In particular, electrochemical sensors are promising tools to detect toxic chemical compounds in industrial products, environmental samples, or biological systems in vivo and in vitro (Hondroulis et al. 2010). Electrochemical impedance spectroscopy (EIS) is an

alternative powerful tool for monitoring the changes in the interfacial properties at the electrode surfaces (Park and Yoo 2003). This technique has been used to detect interactions between biomolecules and cells (Wegener et al. 1999), to develop many kinds of cell sensors (Chen et al. 2005a; Yang et al. 2004), and to analyze toxicity by detecting changes in resistance caused by cells attaching to and spreading on the electrode surface, cell morphology changes, and micromotion in the cell culture (Male et al. 2010; Yoon et al. 2014).

With unique chemical and physical properties, gold nanoparticles (AuNPs) have demonstrated widespread use in fundamental research (Daniel and Astruc 2004; Du et al. 2005), particularly in biological (Hone et al. 2002) and sensing applications (Cao et al. 2002). Gold nanoparticles (AuNPs) are currently the subject of a wide field of research, enhancing the electrocatalytic response of the sensor due to their similarity to a conducting wire or electron-conducting tunnel. In addition, AuNPs also provide additional binding sites for the coupling of a wide range of molecules, including enzymes, antibodies, and cells (Minati et al., 2012).

In recent years, folic acid has been discovered to be an important B class vitamin that specifically targets folate receptors (FR) that are anchored to the cell membrane with a high affinity (K_d of 0.1−1 nM) to facilitate its cellular internalization (Ai et al. 2012; Hu et al. 2009). The binding site to FR is the pteroic groups of folic acid (Chen et al. 2013). FR expression in normal cells/tissues is highly conserved, whereas some malignant tumor cells exhibit overexpressed FR (up to 100−300 times higher than that in normal tissue) due to their high requirement for folic acid (Elnakat and Ratnam

2004).

In these techniques, maintaining cells adhesion and viability on a proper surface invariably constitutes a key step. Type I collagen, which has excellent biocompatibility and high mechanical strength (Krewson et al. 1994), was used to encapsulate BEL-7402 cells and provide a three dimensional (3D) capsule structure for cell proliferation. In this study, the collagen around the cells played a role encapsulating cells not only to maintain their adhesion but also to retain their viability. Collagen gels can be prepared with different pore sizes by varying the concentration for trapping different sized cells (Banerjee et al. 2008).

Therefore, In the present study, we present a novel method of designing a cell-based impedance sensor to evaluate the toxicity of mycotoxins. We aimed to investigate whether the combination of DON and ZEN would enhance their respective cytotoxic potential.

2. Experimental procedures

2.1. Materials and apparatus

Chloroauric acid ($HAuCl_4 \cdot 4H_2O$), p-aminothiophenol (PAPT), folic acid (FA), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), DON and ZEN were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Human hepatocellular carcinoma (BEL-7402) cells were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Type I collagen (rattail, collagen-I) was purchased from BD Biosciences (San Jose, CA, USA). Roswell Park Memorial Institute (RPMI) media and fetal bovine serum

were obtained from Gibco Laboratories (Gaithersburg, MD, USA). All of the solutions were prepared with deionized water, and all of the reagents were of analytical grade. All of the electrochemical experiments were performed with the CHI660e electrochemical workstation (Shanghai, China) using a conventional three-electrode system in a solution containing 2.5 mM of $Fe(CN)_6^{3-/4-}$ and 1.0 M KCl. The morphologies of gold nanocomposites, p-aminothiophenol, folic acid and cells on the gold electrode were characterized using scanning electron microscopy (model Hitachi S-4800, Hitachi Co., Ltd., Tokyo, Japan). BEL-7402 cells were incubated in a CO_2 incubator (Thermo Scientific Forma Series II Water Jacket, Thermo Fisher Scientific Inc., Rockford, IL, USA). The MTT assay was performed using a microplate reader (Spectra MAX 340, Molecular Devices Co., Sunny vale, CA, USA)

2.2 Cell culture

BEL-7402 cells were cultured in a flask in RPMI-1640 medium that was supplemented with 10% fetal calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere containing 5% CO_2. At the growth retardation stage after 3 d, the cells reached the logarithmic phase of growth, after which they were used for experiments.

2.3 Preparation of FA-modified GCE and the immobilization of cells

The glass carbon electrode (GCE) was successively polished with 0.3 μm and 0.05 μm alumina slurry on a polishing cloth, rinsed thoroughly with doubly-distilled water, and then sonicated in ethanol and doubly-distilled water for 10 min. The modification of GCE with AuNPs was achieved using electrodeposition in a

de-aerated precursor solution of 0.5 M H_2SO_4 containing 1 mM of $HAuCl_4$ under a constant potential of −0.25 V for an optimal time of 100 s (Sun et al. 2013), after which the GCE was washed in doubly distilled water. The obtained AuNP-modified GCE was immersed in 20 mmol/L p-aminothiophenol solution for 8 h and then washed in doubly distilled water. The functionalized GCE electrode was then immersed in 5 mM folic acid with a mixture of 0.1 M NHS-0.4 M EDC at room temperature for 4 h and rinsed with deionized water to obtain FA/PATP/AuNP/GCE. Then, 10 μL of a BEL-7402 cell suspension at a concentration of 5×10^5 cells/ml was dropped onto the FA/PATP/AuNP/GCE surface, incubated at 37 °C for 8 h, and then immersed in PBS buffer (pH 7.4) to remove the uncaptured cells. Finally, 5 μL of collagen was dropped onto the electrode to maintain cell adhesion and viability. Before the electrochemical measurements, the modified electrode was cycled between −0.2 and 0.6 V (scan rate, 100 mV s^{-1}) several times until reproducible responses were acquired. The modification of GCE was carried out as described in scheme 1

2.4 Electrochemical measurements

Electrochemical experiments were performed using a CHI660e electrochemical workstation (Shanghai, China) with a conventional three-electrode system comprising a platinum wire as an auxiliary, a saturated calomel electrode as a reference, and a modified electrode with cells packed with collagen as the working electrode. The electrochemical behavior of BEL-7402 cells that were exposed to various concentrations of mycotoxins in PBS (pH 7.4) was studied using electrochemical impedance spectroscopy (EIS) carried out in the presence of 2.5 mM $Fe(CN)_6^{3-/4-}$ and

1.0 M KCl. The impedance spectra were recorded within the frequency range of 10^{-1}–10^{5} Hz. The data of EIS were captured by the ZView software (Jiang et al. 2013). All of the electrochemical experiments were carried out at room temperature. The cytotoxicity of the mycotoxins was calculated as follows:

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

in which R_{FA} is the impedance of the electrode modified with FA, R_{cell} is the impedance of cell immobilized on the electrode, $R_{collagen}$ is the impedance of the cells packed with collagen, and $R_{mycotoxin}$ is the impedance of the cells packed with collagen that were treated with a certain concentrations of mycotoxins (Chen et al. 2005b).

2.5 Statistical analysis

The data are given as mean values ±SEM (standard error of mean). The significance of difference was determined by the unpaired Student's t-test and set at $P < 0.05$. To compare dependent data, an analysis of variance with appropriate post-hoc analyses was made (ANOVA-test, Prophet 5.0 software). To compare the expected values with the measured values, the expected value (in %) was calculated by the addition of the means of, e.g., cytotoxic effect after exposure to one mycotoxin alone with the mean cytotoxic effect of a second mycotoxin:

$$mean_{expected}(\%)=mean_{mycotoxin1}(\%)+mean_{mycotoxin2}(\%) \quad (2)$$

The expected SEM was calculated as follows:

$$SEM_{expected}=[(SEM_{mycotoxin1})^2+(SEM_{mycotoxin2})^2]^{1/2} \quad (3)$$

An additive effect occurs if the measured values do not lie significantly above or below the calculated expected value. If the measured effect lies significantly above

the expected value, then the effect of two substances is potentiating. If the measured effect lies significantly below the calculated (expected) value, then the effect of two substances is antagonistic. The significance of difference was calculated using an unpaired t-test and appropriate tables according to the following equation (Weber et al. 2005):

$$t-\text{test value} = \frac{\left|\text{mean}_{\text{measured}} - \text{mean}_{\text{expected}}\right|}{\left[\left(\text{SEM}_{\text{measured}}\right)^2 + \left(\text{SEM}_{\text{expected}}\right)^2\right]^{1/2}} \quad (4)$$

3. Results and discussion

3.1 Characterization of the modified glassy carbon electrode

The process of electrode modification was described in Scheme 1. In general, AuNPs were uniformly deposited onto the surface of a clean bare GCE (Fig. S1A). This nanocomposite not only enlarged the active surface area of the electrode, but also increased the adsorptive capability to organic molecules. Then, the PATP was adsorbed onto the AuNP-modified GCE through strong coordination interactions between thiol groups and AuNPs. The direct conjugation of PATP with FA occurred through the reaction of the amino groups of PATP with the carboxyl group of FA. The BEL-7402 cells anchored on the modified electrode surface through the high affinity between FA and the folate receptor (FR). Finally, collagen was used to maintain cell adhesion and viability. The morphology of the GCE surface after all steps in electrode fabrication was characterized by SEM imaging (Fig S2).

3.2 Electrochemical characteristics of BEL-7402 cells on a modified electrode

Differential pulse voltammetry (DPV) was used to characterize both the process of constructing the cytosensor and the cells adhesion onto the sensor surface. As shown in Fig. 2A, the current increased when the electrode was modified with AuNPs (curve b) due to its good conductivity and the increased effective area of the working electrode. The modification of PATP on the AuNP/GCE surface resulted in a decrease in the peak current of $Fe(CN)_6^{3-/4-}$. The decreased current response is attributed to the PATP molecules, which dampen the redox reaction. Similarly, the peak currents on the FA/PATP/AuNP/GCE surface were also lower than were those of PATP/AuNP/GCE. When the cells and collagen adhered onto the sensor surface, a decrease in the peak current and a drastic increase in the peak separation were clearly observed, which were ascribed to the blockage of electron transfer by the insulation of the cell membrane and collagen, increasing electrode resistance. We further used EIS to characterize both the process of constructing the cytosensor and cell adhesion onto the sensor surface. The EIS spectra of $Fe(CN)_6^{3-/4-}$ probe showed that different modified electrodes have different electron-transfer resistance (R_{et}) values (Fig. 2B). With the bare GCE, the R_{et} value was 1154. After the formation of AuNPs, the R_{et} decreased greatly to 40.1 due to its favorably high electrical transport properties. When AuNPs were modified with PAPT, the R_{et} increased significantly from 40.1 to 464.2. When FA was conjugated with PAPT, the R_{et} further increased to 2080. When BEL-7402 cells were attached to the FA/PATP/AuNP/GCE, the barrier near the electrode surface

hampered the redox probe and thus increased the R_{et}; when the collagen encapsulated the cells, the R_{et} increased to 9192. These results of EIS are similar to the changes in DPV responses. Compared to differential pulse voltammetry, EIS is a noninvasive method, producing highly sensitive signals. Therefore, EIS was used to monitor the proliferation and apoptosis of BEL-7402 cells on electrode surface.

3.3 Electrochemical characteristics of the effect of FA for capturing BEL-7402 cells specifically

In order to investigate the effect of FA on capturing BEL-7402 cells specifically, we used PATP/AuNP/GCE-modified electrode as a control. The other electrode was a FA/PATP/AuNP/GCE-modified electrode. A total of 10 μL of a BEL-7402 cell suspension at a concentration of 5×10^5 cells/ml was dropped onto the surfaces of both electrodes, incubated at 37 °C for 8 h, and then immersed in PBS buffer (pH 7.4) to remove the uncaptured cells. From the figure, we can see clearly that the R_{et} of PATP/AuNP/GCE-modified electrode increased from 618 to 1563 (Fig. 3A). FA can capture BEL-7402 cells specifically, which acted as a barrier to the interfacial electron transfer, thus leading to an increase in the R_{et} from 2179 to 6595 (Fig. 3B). BEL-7402 cells have been proved to exhibit overexpressed FR, the activity of FR is related to the viability. The dead cells may affect the interaction between folic acid and folate receptor(Hong et al. 2012). During the experiment, we also find that the impedance of alive cells is more than the dead cells. Maybe due to the activity of folate receptor of dead cells decreased, the dead cells can not be anchored on the electrode through the interaction between folic acid and folate receptor. Compared with using collagen or

other biological affinity materials to immobilize the cell onto the electrode surface directly, folic acid can bind to these receptors of the hepatoma carcinoma cells, which have good viability.

3.4 Electrochemical characteristics of the effect of collagen to maintain cell adhesion and viability

In order to investigate the effect of collagen on maintaining cell adhesion and viability, we used the Cell/FA/PATP/AuNP/GCE-modified electrode with collagen as control and another modified electrode without collagen. Both electrodes were immersed in PBS (pH 7.4). From Fig. 3C, we can clearly see that, with time increased from 0 h to 60 h, the EIS of the control decreased slightly; however, the EIS of the modified electrode without collagen decreased significantly (Fig. 3D). The impadance of the modified electrode is related to the cell number and viability. This indicated that the modified electrode without collagen shows decreasing the viability of cells. It is possible that due to nutrient shortage after a long period decreasing the viability of the cells, some cells dropped off the electrode. This phenomenon suggests that collagen can supply the cells with nutrition, maintaining adhesion and viability.

3.5 Effect of DON and ZEN and their combination on the viability of BEL-7402 cells

The electron-transfer resistance was related to not only the amount of cells that were immobilized onto the modified electrode but also to the proliferation and apoptosis of the adhered cells. Thus, in addition to the detection of cell immobilization, this method could be used to monitor both the proliferation and

apoptosis of cells on electrode surface in real time. Typical impedance results are presented in Nyquist diagrams (Fig. 4). The Nyquist diagram, however, indicates toxicity, as shown by decreased R_{et} values, suggesting decreased BEL-7402 cell proliferation. When the modified electrodes were exposed to 5 μg/ml DON, 10 μg/ml ZEN and their combination; a notable change in the impedance signal of the BEL-7402 cells was observed. The presence of mycotoxins decreased the impedance that was recorded for BEL-7402 cells, indicating a reduction in the viability of these cells. Compared with the MTT assay, the detailed operation of which was put in the supporting informations, the electrochemical method was simple and convenient which can evaluate the viability of cells by electrochemical impedance spectroscopy. However, the conventional methods was time-consuming and complex which can only assess the viability of cells at one time point. Our purpose is to investigate the proposed method can assess toxicity with long-term monitoring and real-time measurements. When the culture time increased from 6 h to 30 h, the impedance of the BEL-7402 cells decreased significantly, especially when exposed to the combination of both DON and ZEN. Because in vitro toxicity studies on trichothecenes revealed that DON binds to the ribosomal peptidyltransferase site and inhibits protein synthesis, resulting in decreased cell proliferation (Shifrin and Anderson 1999). ZEN induced several genotoxic effects in vitro and in vivo (Ouanes et al. 2003). Oxidative damage is likely to be evoked as one of the main pathways of ZEN toxicity (Hassen et al. 2007). Thus, oxidative stress is implicated in both DON and ZEN toxicities. In this study, we also investigated the binary mixtures of ZEN and

DON on ROS production (Fig. S5).

3.6 Electrochemical mycotoxin sensitivity test

To analyze the individual toxicity of DON and ZEN on BEL-7402 cells, the modified electrode was exposed to different concentrations of DON and ZEN for 24 h. Impedance changes due to cell proliferation and apoptosis on the electrode surface were measured three times after the treatments. The subsequent change in the electrochemical response of the BEL-7402 cells enabled the cytotoxicity of the different mycotoxins to be evaluated. Fig 5A、B shows the cytotoxicity curves for 24 h exposure to these mycotoxins, which were applied within suitable concentration ranges. The proposed electrochemical mycotoxin sensitivity test and the MTT assay of BEL-7402 cells showed an increasing cytotoxicity of these mycotoxins as the concentration increased. These data indicate that both DON and ZEN reduce BEL-7402 cell viability as expected; however, DON is more effective in reducing cell viability, whereas ZEN has weak cytotoxicity. As the concentration of DON increased from 0.1 μg/ml to 20 μg/ml, the inhibition rate of BEL-7402 cells significantly increased from 1.2% to 61% as measured by EIS. The inhibition rate increased from 0.7% to 60% as measured by EIS, after treatment with 0.1 μg/ml to 50 μg/ml of ZEN. This conclusion was confirmed by the MTT assay. We can clearly see from the inserted figure, DON and ZEN reduced cell survival in a dose-dependent manner, especially at low concentions, when the cells treated with various concentrations of deoxynivalenol (from 0.1 to 2 μg/ml), zearalenone (from 0.1 to 10 μg/ml) showed a quasi-linear relationship. The detection limt of deoxynivalenol and zearalenone was

0.03 and 0.05 μg/ml, respectively. As the concentration of mycotoxin was increased , the quasi-linear behavior of the EIS disappeared. It is possible that the effects of the mycotoxin were limited by the concentration of cells. What's more, the proposed method was more sensitive than MTT assay at low concentrations. The mycotoxin concentration corresponding to the cytotoxicity of half of the maximum cytotoxicity was defined as IC_{50}, which for DON as obtained by EIS and MTT assay was 7.1 and 9.3 μg/ml, respectively, while for ZEN was 24.6 and 27.2 μg/ml, respectively. Clearly, the IC_{50} values that were obtained using the proposed electrochemical method were very close to those that were obtained using the MTT assay.

To estimate the cytotoxic interactions of DON and ZEN, six concentrations of DON (0.1, 0.2, 0.5, 1, 2 and 5 μg/ml) and ZEN (0.1, 0.2, 0.5, 1, 5 and 10 μg/ml) below IC_{50} were used (Fig. 5C). After exposure to DON+ZEN at 0.1+0.1, 0.2+0.2, 0.5+0.5, 1+1, 2+5 and 5+10 μg/ml for 24 h, We found that every combination of these mycotoxins induced a significant decrease in the level of cell death compared to the effect of mycotoxins taken separately. The results that were obtained by the proposed electrochemical mycotoxins sensitivity test and the MTT assay were very close. Comparing the measured results with the expected results of the EIS and MTT assay, respectively, the difference in the combinations was not significant, indicating an additive effect. In general, the electrochemical cytotoxicity evaluation results agreed with the results of MTT assays; the slight difference might be attributed to the different culture conditions and assay procedures. We also investigated the effects of mycotoxins on $[Ca^{2+}]_i$ production (Fig S6) and apoptosis rate (Fig S7) in BEL-7402

cells, which indicated that the proposed electrochemical method is a credible sensitivity test for mycotoxin. In this study, not every possible combination of toxins was tested; this demand would have been beyond the scope of this study. However, we demonstrated that with our method, a rapid and cost-efficient possibility exists to test combinations of mycotoxins of interest and to obtain a first glimpse of their toxic potentials.

4. Conclusion

The electrochemical mycotoxin sensitivity test offers potential advantages over conventional assays. With EIS measurements, the cell-based electrochemical biosensor can be used to evaluate the individual and combined toxicity of DON and ZEN on BEL-7402 cells. Our results show that DON and ZEN caused a marked decrease in the cell viability in a dose-dependent manner. The value of electrochemical impedance spectroscopy decreased with the concentration of DON and ZEN in range of 0.1-20, 0.1-50 μg/ml with the detection limit as 0.03, 0.05 μg/ml, respectively, the IC_{50} for DON and ZEN as obtained by the proposed electrochemical method was 7.1 μg/ml and 24.6 μg/ml, respectively, and the combination of the two mycotoxins appears as an additive response. The electrochemical approach is highly sensitive, real time, accurate and simple, which could be developed as a convenient means to study the toxicity of mycotoxins. The RSD of the detection results was all lower than 5%, which indicated that modification electrode has good reproducibility and stability. While more regeneration times and long-term storage of modified electrode need more experiments to verify. Furthermore, the proposed method could

be used to realize high-throughput analysis in future. To our knowledge, our study is the first to present a novel method of designing a cell-based impedance sensor to evaluate the combined toxicity of DON and ZEN.

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Highlight

1. folic acid was used to immobilize cells with good viability on the electrode.
2. This method can assess the toxicity with long-term monitoring and real-time measurements.
3. First time to use electrochemical method to evaluate the combined toxicity of mycotoxins.

Fig captions

Scheme 1. Schematic illustration of the preparation of modified electrode. Inset: SEM images of cells with collagen on the modified electrode.

Fig 2. (A) Differential pulse voltammetry of bare GCE (a), AuNP/GCE (b), PATP/AuNP/GCE (c), FA/PATP/AuNP/GCE (d), Cell/FA/PATP/AuNP/GCE (e), Collagen/Cell/FA/PATP/AuNP/GCE (f). (B) Nyquistdiagrams of electrochemical impedance spectra of bare GCE (a), AuNP/GCE (b), PATP/AuNP/GCE (c), FA/PATP/AuNP/GCE (d), Cell/FA/PATP/AuNP/GCE (e), Collagen/Cell/FA/PATP/AuNP/GCE (f) in $Fe(CN)_6^{3-/4-}$, scan rate: 100 mV s^{-1}.

Fig 3. Electrochemical impedance spectroscopy (EIS) of (A): PATP/AuNP/GCE (a) Cell/ PATP/AuNP/GCE (b) and (B): FA/PATP/AuNP/GCE (a) Cell/FA/PATP/AuNP/GCE (b).

Electrochemical characteristics of the effect of collagen, as culture time increased, nyquist diagrams of (C) the Cell/FA/PATP/AuNP/GCE modified electrode with collagen and (D) the modified electrode without collagen. (a) 0 h, (b) 12 h, (c)24 h, (d)36 h, (e)48 h, (f) 60 h.

Fig 4. Effect of (A)DON (5 μg/ml) (B)ZEN (10 μg/ml) and (C)their combination on the viability of BEL-7402 cells for different time. (a) 0 h, (b) 6 h, (c) 12 h, (d) 18 h, (e)24 h, (f) 30 h. (D): The impedance of the cells on the modified electrode after exposed to the mycotoxins as time increased.

Fig 5. Cytotoxicity curves for 24 h exposure of the BEL-7402 cells to DON and ZEN obtained by using the MTT assay and the proposed electrochemical method. (A) DON :0.1 μg/ml, 0.2μg/ml, 0.5 μg/ml, 1 μg/ml, 2 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml. (B) ZEN: 0.1 μg/ml, 0.2 μg/ml, 0.5 μg/ml, 1 μg/ml, 5 μg/ml, 10 μg/ml, 20 μg/ml, 50 μg/ml. (C) Cytotoxicity effect of combined toxicity of DON+ZEN: 0.1+0.1 μg/ml, 0.2+0.2 μg/ml, 0.5+0.5 μg/ml, 1+1 μg/ml, 2+5 μg/ml, 5+10 μg/ml on BEL-7402 cells after 24 h incubation evaluated by EIS and MTT assay. Gray and dark bars represent the expected (calculated) and measured results of EIS and MTT assay respectively. *Indicates significant antagonisation or potentiation ($p < 0.05$) of cytotoxicity effect.

Scheme.1

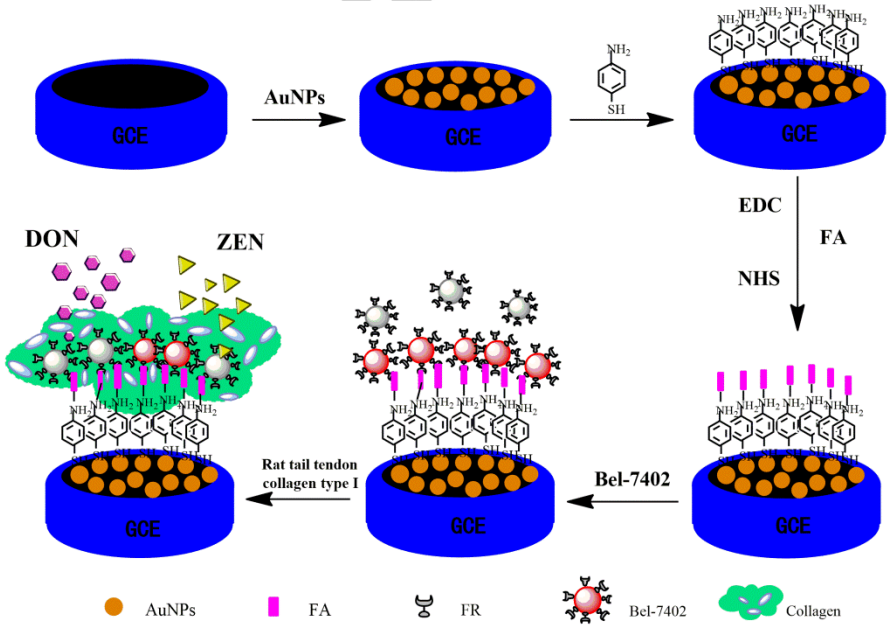

Fig.2

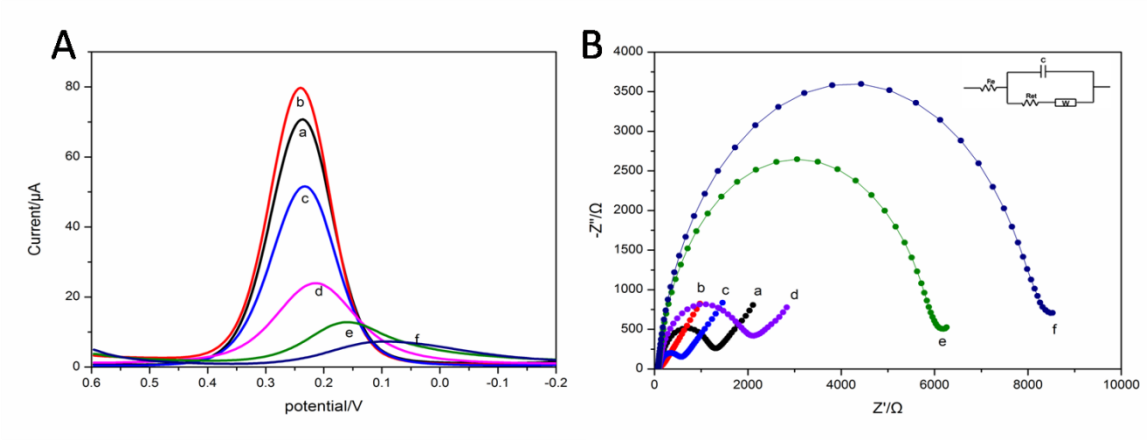

Fig.3

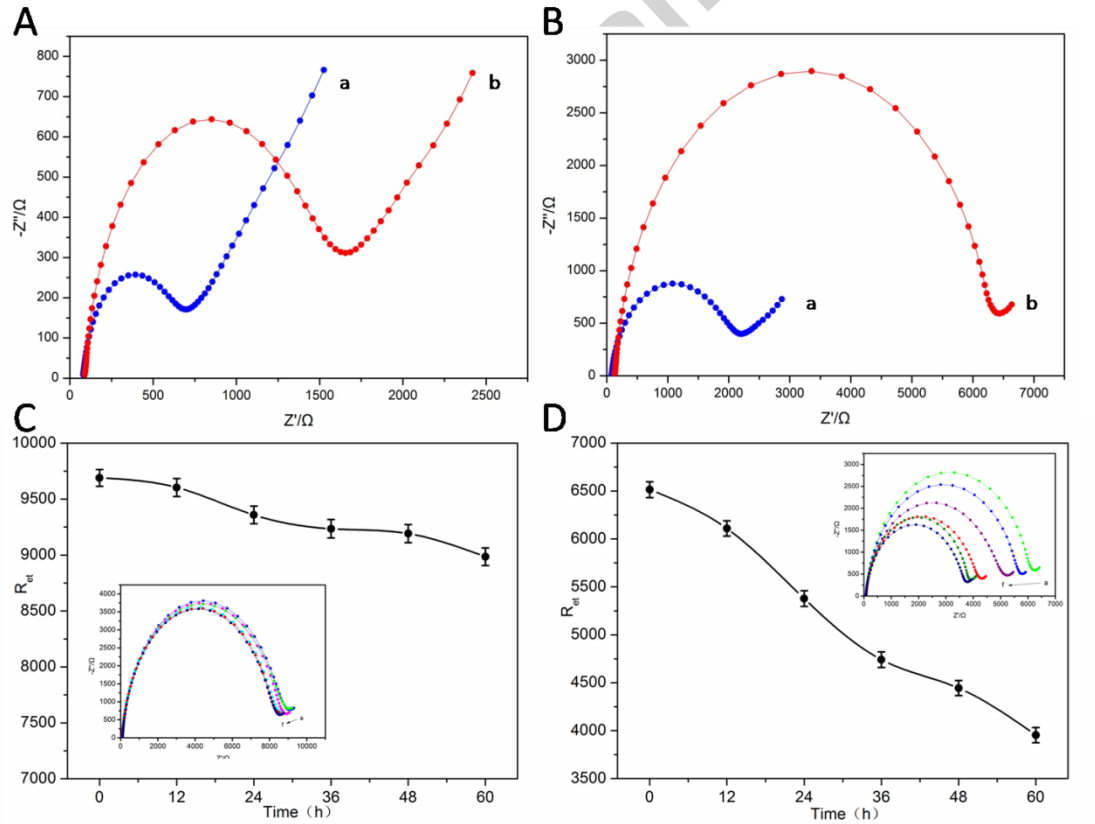

Fig.4

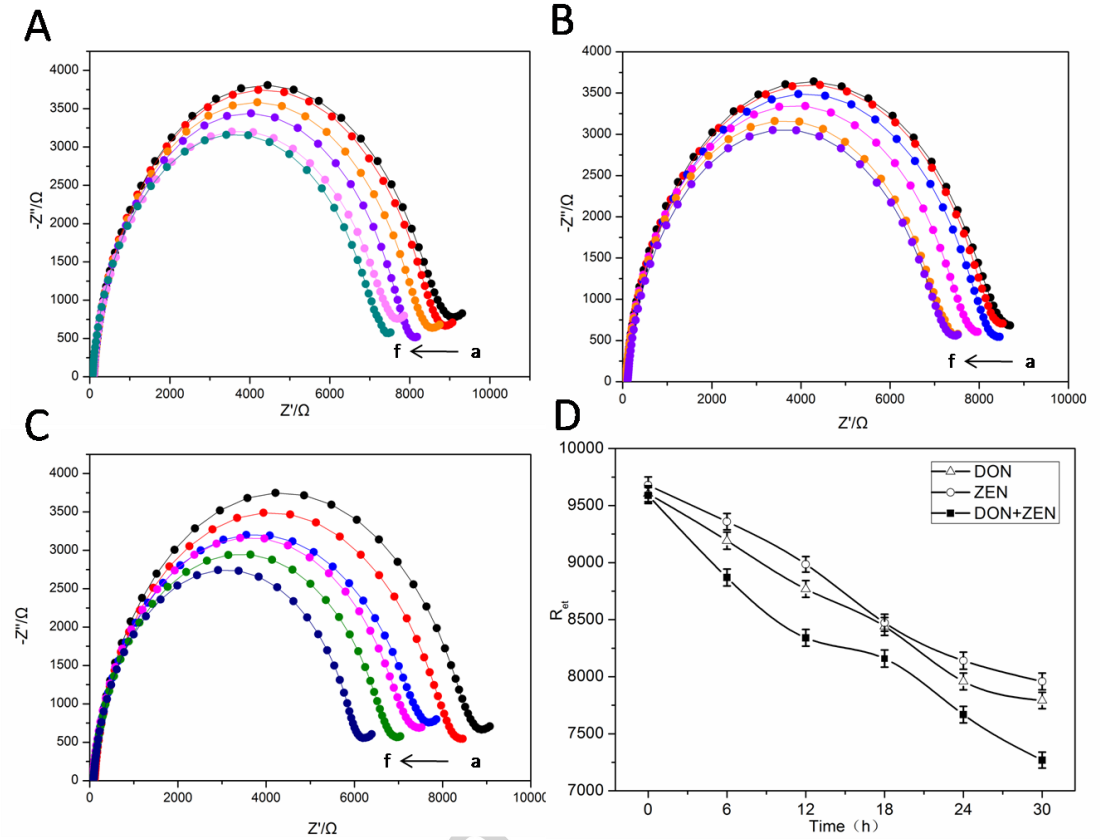

Fig.5