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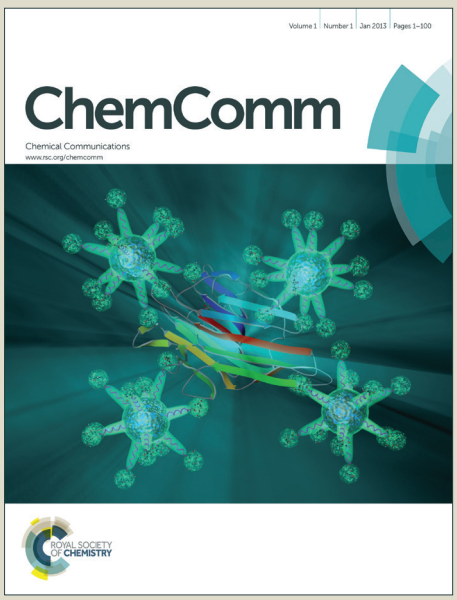

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Transient transmembrane secretion of H_2O_2: A mechanism for citral-caused inhibition of aflatoxin production from *Aspergillus flavus*

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A polydopamine-Fe_3O_4 nanocomposite-based H_2O_2 electrochemical sensor is fabricated to real-time monitor the transmembrane release of reactive oxygen species from citral-treated *Aspergillus flavus*, revealing a mechanism involving transient transmembrane secretion of H_2O_2 for citral-caused inhibition of aflatoxin production from fungus for the first time.

Aflatoxins are a group of highly toxic and carcinogenic metabolites that are produced by *Aspergillus flavus* and *A. parasiticus*.[1] A low quantity (parts per billion) of aflatoxins can lead to significantly negative impacts on human and animal health.[2] Aflatoxin-producing fungi are widely spread in nature and can contaminate food and feed at each procedure of grain production.[3] How to reduce the generation of aflatoxins from the fungi is one of the hottest research topics in microbiology. The use of natural essential oils for the protection of grains has been regarded as a facile, safe and effective way to restrain the fungi growth and aflatoxin production.[4] As a typical natural essential oil presented in various plants, citral shows strong inhibitory effects on aflatoxin production and is proposed to be a very promising substance for the control of aflatoxins in grains.[5] However, existing studies are limited to the dose-response of citral and aflatoxin production. The mechanism for citral-induced inhibition on aflatoxin biosynthesis in fungus is still obscure.

Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radical are continuously generated during cellular metabolic processes. They act as signaling molecules in the maintenance of cellular normal physiological functions.[6] The involvement of ROS in the generation of aflatoxins has also been reported in previous research works.[7] The enhancement of oxidative stress may result in the production of aflatoxins from toxigenic strain.[8] Furthermore, the application of antioxidants could repress the biosynthesis of aflatoxins from *Aspergillus* species.[9] Thus, ROS could play an important role in citral-caused inhibition on aflatoxin production.

H_2O_2 is one of the most stable ROS with a half-life of several hours, which could diffuse across membranes through water channels.[10] In recent years, electrochemical sensors have been employed to real-time monitor H_2O_2 in biological systems due to their simplicity, fast response, high sensitivity and low detection limit.[11] In the present work, we fabricated a polydopamine (PDA)-Fe_3O_4 nanocomposite based non-enzymatic H_2O_2 sensor to real-time monitor the H_2O_2 release from citral-treated *A. flavus* cells. The intracellular oxidative stress, morphological changes and the aflatoxin B_1 (AFB_1) production of *A. flavus* mycelia were also measured to reveal the role of H_2O_2 in citral-induced aflatoxin suppression.

Fe_3O_4 nanoparticles (NPs) were decorated on PDA microspheres via a facile *in situ* co-precipitation method and the as-prepared nanocomposites were characterized with transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Fig. 1). In comparison to the smooth surface of the PDA microspheres (Fig. S1 in ESI), a lot of small dots with the size less than 10 nm are discovered, suggesting the successful growth of Fe_3O_4 NPs in the nanocomposites. The average size of the PDA-Fe_3O_4 nanocomposite is ~500 nm. The surface attached NPs are of single-crystalline structures with lattice spacing distances of 0.25 and 0.30 nm, corresponding to the (311) and (220) planes of the face-centered cubic Fe_3O_4, respectively. The excellent crystalline structure (Fig. S2 in ESI) of the NPs could guarantee the electrocatalytic activity of the nanocomposites. The abundant surface chemical groups of PDA render the nanocomposites hydrophilic for the easy access of H_2O_2 molecules in the following sensing applications (Fig. S3 in ESI).

PDA-Fe_3O_4 nanocomposites were modified on a glassy carbon electrode (GCE) to fabricate an electrochemical sensor towards H_2O_2 reduction. Cyclic voltammetry (CV) was employed to characterize the catalytic behavior of the sensor over a potential ranging from −0.9 to +0.2 V at a scan rate of 50 mV/s in pH 7.4

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phosphate buffer saline (PBS) with bare GCE, PDA microspheres-immobilized GCE (PDA/GCE) and Fe_3O_4 NPs-functionalized GCE (Fe_3O_4/GCE) for comparisons (Fig. 1D). The nanocomposite-base electrode shows a well-defined reduction peak with the highest current intensity in a 0.01 M PBS (pH 7.4) containing 1.0 mM H_2O_2, verifying the catalytic activity of Fe_3O_4 NPs to the reduction of H_2O_2. Fig. 1E illustrates a typical amperometric response of the sensor on successive addition of H_2O_2. The catalytic currents reach the steady-state value (95% of the maximum) within 5 s after each H_2O_2 injection. The fast response of the sensor can meet the requirements of cell-released H_2O_2 measurement. The currents show a linear response against the H_2O_2 concentration in the range of 0.5 to 6.0 μM（R^2=0.995）with a sensitivity of 51.06 μA/mM and a detection limit of 4.9×10^{-8} M (S/N = 3). In comparison with the reported non-enzymatic H_2O_2 sensors, the PDA-Fe_3O_4 based one has a comparable sensitivity and a lower detection limit (Table S1 in ESI). Inset II of Fig. 1E illustrates the great selectivity of the sensor. The hydrophilicity of PDA and the well-dispersibility of Fe_3O_4 NPs in the nanocomposites may contribute to the excellent performance of the sensor. A micromolar level of steady-state extracellular H_2O_2 could be generated in a biological system with the cell density of 10^5/mL.[12] The results in this assay confirm that the PDA-Fe_3O_4 nanocomposite-based H_2O_2 sensor can be applied to monitor the secretion of H_2O_2 in an *A. flavus* mycelia suspension system.

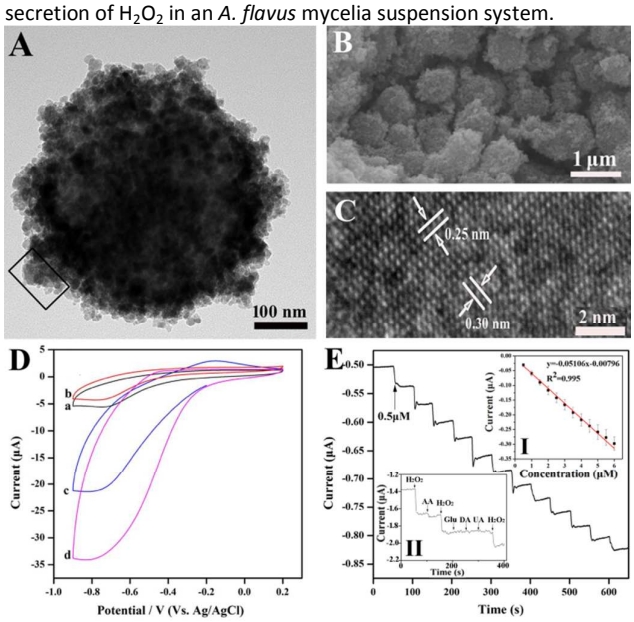

Fig. 1 (A) TEM image of the PDA-Fe_3O_4 nanocomposites; (B) SEM image of the PDA-Fe_3O_4 nanocomposites; (C) HRTEM image of the Fe_3O_4 nanoparticles on the PDA-Fe_3O_4 nanocomposites; (D) CV of bare GCE (a), PDA/GCE (b), Fe_3O_4/GCE (c) and PDA-Fe_3O_4-Nafion/GCE (d) in a 0.01 M PBS buffer (pH 7.4) containing 1.0 mM H_2O_2 with a scan rate of 50 mVs^{-1}; (E) Amperometric response of PDA-Fe_3O_4-Nafion/GCE on successive injection of H_2O_2 into a 0.01 M PBS buffer (pH 7.4). Applied potential: -0.3 V. Inset I: plot of H_2O_2 peak current versus H_2O_2 concentration. Inset II: Interfering effect of ascobic acid (AA), glucose (Glu), dopamine (DA) and uric acid (UA) on the performance of PDA-Fe_3O_4-Nafion/GCE.

To verify the role of ROS in citral-induced suppression of aflatoxin production, the secretion of H_2O_2 from *A. flavus* mycelia with/without citral treatment was real-time measured using the as-prepared electrochemical sensor. In our investigation, it is found that the amount of aflatoxins in the conidia-inoculated culture medium explosively grows in the incubation time range of 60 to 108 h(Table S2 in ESI). Thus, mycelia after 60 h of culture were utilized in the following experiments. As being exhibited in curve (a) (Fig. 2), the addition of 300 ppm citral in the PBS without *A. flavus* mycelia does not cause any electrochemical response. 30% glycerin, the solvent of citral, also has no significant impact on the current in a mycelia suspension (curve b of Fig. 2). The data rule out the interference of stimulator in the electrochemical detection at the potential of -0.3 V versus Ag/AgCl. A remarkable enhancement of the reduction current is obtained in a PBS (0.01 M, pH 7.4) containing mycelia after the injection of 300 ppm citral (curve c in Fig. 2). Moreover, the introduction of catalase (100 U mL^{-1}), a selective scavenger of H_2O_2, can induce the recovery of the current back to the background level, indicating that the increase of current is ascribed to citral-induced secretion of H_2O_2 from the mycelia. According to the calibration curve in Fig. 1E, the amount of the released H_2O_2 is calculated as 304.4 nM (the dry weight of mycelia is 0.2744 g). The data clearly show that citral-treatment could trigger the release of H_2O_2 from *A. flavus* cells. To the best of our knowledge, it is the first time to real-time monitor the section of H_2O_2 from fungi cells. It has been reported that citral can cause significant damage to cell membrane and cell wall of microbes.[13] Thus, the citral treatment may also destroy the cell membrane and cell wall structure of *A. flavus* mycelia, consequently resulting in the H_2O_2 secretion.

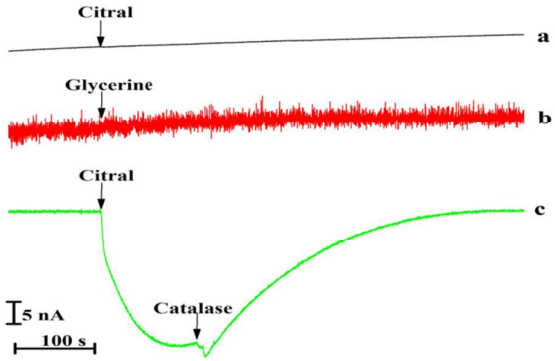

Fig. 2 (a) Amperometric response of the sensor in a PBS solution without *A. flavus* cells under citral injection; (b) Amperometric response of the sensor in a PBS solution containing *A. flavus* cells under injection of glycerine, the solvent for citral; (c) Amperometric response of the sensor in a PBS solution containing *A. flavus* cells treated with citral (300 ppm), followed by a catalase injection (100 U mL^{-1}).

The secretion of H_2O_2 may cause the decrease of intracellular ROS level, further leading to the alleviation of oxidative stress in the mycelia. Since the formation of oxidative stress is a pre-requisite for the biosynthesis of aflatoxins,[8] it is of great importance to evaluate the intracellular oxidative stress after the citral-treatment. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay was employed to

show the intracellular oxidative stress in the present study (Fig. 3A and 3B). The glycerine-treated mycelia possess high intensity of green fluorescence, but the citral-treated ones demonstrate less fluorescence emission. The results indicate that the ROS level in the former samples is much higher than that in the latter ones. Therefore, the citral-induced section of H_2O_2 may reduce the amount of ROS in the mycelia, subsequently resulting in the alleviation of oxidative stress. Interestingly, it is observed that the average diameter of the citral-treated mycelia is smaller than that of glycerine-exposed ones (Fig. 3C and 3D). A lot of wrinkles can also be found on the surface of citral-treated mycelia. The findings prove the occurrence of the cellular secretion process and may suggest the co-release of other intracellular substances like H_2O molecules together with H_2O_2. Vacuoles in *A. flavus* cells are correlated to the biosynthesis of aflatoxins,[14] thus the citral-caused secretion may also possibly inhibit the aflatoxin production via the change of vacuole volume.

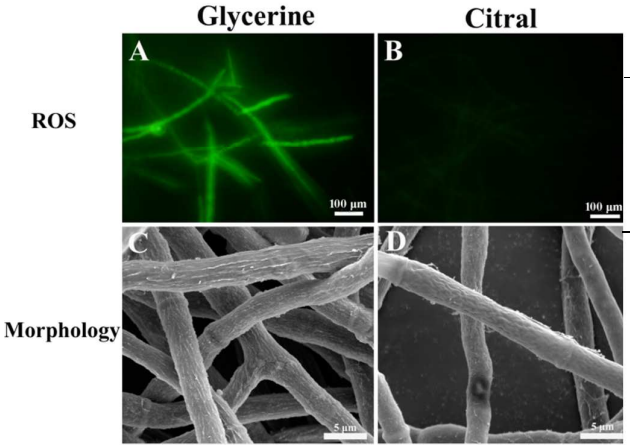

Fig. 3 Effects of citral treatment on the intracellular ROS level and surface morphology of mycelia. Fluorescent micrographs of the mycelia treated with glycerine (A) and citral (B), respectively; SEM images of the mycelia treated with glycerine (C) and citral (D), respectively.

After citral- or glycerine-treatment the *A. flavus* mycelia were harvested and cultured in a freshly prepared medium subsequently. The amount of AFB_1 in the culture medium was measured after 0, 24 and 48 h of incubation at 30 °C to evaluate the effects of citral on the production of aflatoxins (Table 1). Initially, the concentration of AFB_1 in both glycerine- and citral-treated groups is around 200 ng mL^{-1}, which may be attributed to desorption of mycelium surface-adsorbed aflatoxin molecules. After 24 h of incubation only 8.2 ± 3.5 ng mL^{-1} of AFB_1 increment is detected in the citral group. However, the enhancement of AFB_1 concentration in the glycerine samples is 32.8 ± 4.9 ng mL^{-1}. From 24 to 48 h a sharp increase on the concentration of AFB_1 occurs in the glycerine-exposed group. The accumulated AFB_1 concentration reaches to 1689.4 ± 2.4 ng mL^{-1} with an increment of 1439.9 ± 4.9 ng mL^{-1}. The high efficient biosynthesis of aflatoxin after 24 h of culture may lead to the tremendous growth of AFB_1 concentration. In contrast, the citral-treated samples show an enhancement of 145.4 ± 1.0 ng mL^{-1} on the AFB_1 level, which is around 10 times lesser than the one observed in the glycerine group. The results strongly support that a short-term exposure of *A. flavus* mycelia to citral could effectively diminish the generation of aflatoxins. The amount of AFB_1 released from the citral-treated mycelia may continuously increase after 48 h. However, its growth should be much slower than that of glycerine-exposed ones. The influence of long-term citral treatment on the production of aflatoxin has been well documented in the literatures.[15] It is the first time to report the effective control of aflatoxins via a short-term citral exposure, which undoubtedly should be a safer and more economic approach. However, it should be noted that the treatment timing is very critical for the outcome of this strategy. Based on our data, the citral should be applied at least 24 h before the explosive production of aflatoxins.

Table 1 Generation of AFB_1 from the glycerine- and citral-treated mycelia

Time	Glycerine		Citral	
(h)	AFB_1 (ng/mL)	ΔAFB_1 (ng/mL)	AFB_1 (ng/mL)	ΔAFB_1 (ng/mL)
0	216.6±2.3	-	196.2±2.0	-
24	249.5±2.5	32.8±4.9	204.4±1.5**	8.2±3.5**
48	1689.4±2.4	1439.9±4.9	349. 8±2.4**	145.4±1.0**

**: p<0.01, compared with the glycerine-treated mycelia.

The short-term citral treatment caused inhibition of AFB_1 production in *A. flavus* mycelia could be explained as the follows. Normally, after certain duration of culture ROS gradually accumulate in the premature mycelia to trigger a series of biochemical reactions for the aflatoxin biosynthesis. However, upon the addition of citral large amount of ROS is secreted from the mycelia, further resulting in the decrease of intracellular ROS level. The failure formation of oxidative stress interrupts the signaling pathway to the aflatoxin biosynthesis. Therefore, the production of aflatoxins from citral-incubated *A. flavus* mycelia is inhibited significantly. More works are still carrying out in the authors' laboratory to elucidate the detailed pathway.

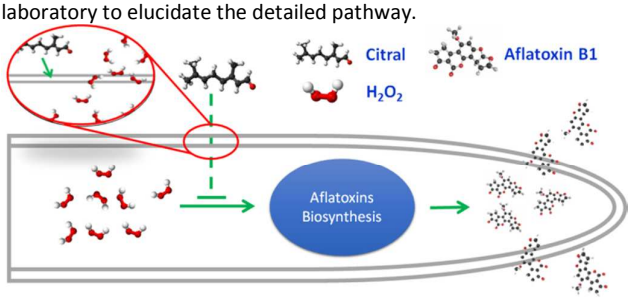

Fig. 4 Mechanism for citral-caused inhibition of aflatoxin production in *A. flavus*.

In summary, a PDA-Fe_3O_4 nanocomposite-based highly sensitive electrochemical sensor was fabricated for real-time monitoring the secretion of H_2O_2 molecules from *A. flavus* mycelia after short-time treatment with citral. The real-time measurement results associated with the intracellular ROS level and morphological changes of the mycelia prove the transit transmembrane release of

H_2O_2 in the citral exposure process. The remarkable inhibition effect of citral on AFB_1 production was also demonstrated by culturing the treated mycelia for another 48 h. A mechanism involving the transient secretion of H_2O_2 from the mycelia is proposed to explain citral-caused inhibition of aflatoxin production from *A. flavus*.

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