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Electron coupling effect-triggered monatomic copper laccase-mimicking nanozyme for the degradation and detection of guaiacol produced by *Alicyclobacillus acidoterrestris*

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ABSTRACT

The generation of guaiacol by *Alicyclobacillus acidoterrestris* (*A. acidoterrestris*) in fruit juices negatively affects public health and causes severe environmental pollution. Therefore, the sensitive detection and efficient degradation of guaiacol in real samples are crucial. Here, we develop an electrochemical sensor utilizing a copper single-atom nanozyme (CuN₄-G) to detect and degrade guaiacol at the picomolar level. Density functional theory (DFT) calculations verify that the bonding electron coupling effect in the CuN₄-G facilitates rapid electron transfer, enhances electrical conductivity, and provides abundant active sites, thereby leading to exceptional catalytic performance. Moreover, CuN₄-G demonstrates a K_m value similar to that of natural laccase but a higher V_{max} , highlighting its potential as a highly efficient biocatalyst. The CuN₄-G-based electrochemical sensor achieves a detection from 5 to 50,000 pM for guaiacol, with a 1.2 pM (S/N = 3) detection limit. Additionally, CuN₄-G-modified electrodes display high selectivity and excellent stability. CuN₄-G nanozyme can keep its activity in conditions of pH (3–9), temperature (30–90 °C), ionic strength (0–400 mM), and organic solvent (0–50% (v/v)), overcoming the deficiencies of natural enzymes. Furthermore, our electrochemical sensor can not only accurately detect guaiacol, but also degrade it in actual fruit juice samples infected by *A. acidoterrestris*, demonstrating its potential applications in food and environmental monitoring.

1. Introduction

Guaiacol, a compound with aromatic characteristics, is widely distributed in plants and can also be found in various food sources such as smoked food (Shishov et al., 2020), wine (Martinez Enriquez et al., 2009), and beverages (Jia et al., 2022). There are increasing reports that guaiacol as the primary metabolite is produced by *A. acidoterrestris* existing in fruit juice (Hu et al., 2020). Due to the remarkable resistance of *A. acidoterrestris* to both heat and acidic conditions, it can persist even in pasteurized beverages, significantly impacting the flavor profile of such beverages (Shi et al., 2021; Thi Song Van et al., 2021). In apple juice and orange juice, guaiacol concentrations exceeding 2 ppb can result in the development of a distinct "smoky" or "medicinal" off-flavor (Ruiz Perez-Cacho et al., 2011; Siegmund and Pollinger-Zierler, 2006). Furthermore, excessive ingestion of guaiacol can pose substantial risks to liver and kidney health (Martinez Enriquez et al., 2009). Therefore,

achieving accurate determination and effective degradation of guaiacol in food products hold paramount importance in the field of biophysical chemistry.

To date, several detection approaches, including solid-phase microextraction-gas chromatography-mass spectrometry (Guggenberger et al., 2019), gas chromatography-mass spectrometry (Pardo-Garcia et al., 2017), high-performance liquid chromatography-tandem mass spectrometry (Hayasaka et al., 2010), electronic nose (Huang et al., 2015), have been developed to analyze guaiacol. In comparison to these methods, electrochemical techniques offer a promising alternative due to their rapid analysis, cost-effectiveness, simplicity, and high sensitivity (Sun et al., 2015). For instance, there are various enzyme sensors reported with the redox enzymes activity such as laccase activity (Othman and Wollenberger, 2020), horseradish peroxidase activity (Yan et al., 2021), and tyrosinase activity (Wu et al., 2020a) for phenolic compound detection.

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Scheme 1. Schematic of electrochemical sensor based on monatomic copper laccase-mimicking nanozyme for detection and degradation of guaiacol in food.

Laccase, an enzyme that facilitates the oxidation of phenolic substrates while reducing molecular oxygen by accepting four electrons, has garnered extensive attention because of its eco-friendly nature for phenolic degradation (Jones and Solomon, 2015; Latif et al., 2022; Mate and Alcalde, 2017; Senthivelan et al., 2016; Taghizadeh et al., 2020). However, its practical application is hindered by challenges related to high production costs and limited stability (Ballesteros et al., 2021; Jiang et al., 2019; Makam et al., 2022; Romanholo et al., 2021; Wang et al., 2019a). In this case, developing enzyme mimics with superior catalytic activity, excellent stability, and cost-effectiveness, will overcome these limitations and expand the usefulness of enzymes. For instance, a nanozyme with laccase-like activity based on guanosine monophosphate coordinated copper was successfully constructed by Liang et al., (2017). A nanozyme as laccase mimic through the coordination of Cu⁺/Cu²⁺ with a cysteine (Cys)-histidine (His) dipeptide was designed by Wang (Wang et al., 2019b). Additionally, Makam et al. demonstrated the development of a bionanozyme by the supramolecular assembly of phenylalanine and Cu^{2+} (Makam et al., 2022). While these biomimetic enzymes exhibit equal or even higher catalytic performance than natural laccases, many of them rely on intricate synthetic method and involve complex and costly ligand molecules. Therefore, it is essential to design nanozyme materials that are simple and economical, aiming to address these challenges and promote wider practical applications.

Single atom catalysts (SACs), initially reported for their exceptional catalytic performance in 2011, have been extensively explored in electro-catalysis (Cheng et al., 2019; Qiao et al., 2011). This is attributed to their maximum atoms' utilization, simple synthetic strategies, and excellent catalytic activities (Wang et al., 2020; Xi et al., 2021). Nevertheless, high surface free energy of these isolated single atoms often makes them prone to aggregation during synthesis processes (Fei et al., 2019; Yang et al., 2013). Thus, the isolated atoms are usually immobilized on appropriate supports, endowing them the dispersion and stability of the isolated active atoms through metal-support interactions (Zhu et al., 2017). Graphene, with its ultrahigh theoretical surface area, excellent electrical conductivity, and extraordinary chemical and electrochemical stability (Alwarappan et al. 2009, 2010, 2012), has emerged as an attractive substrate for many SACs (Wang et al., 2010; Zhuo et al., 2020). Additionally, metal-nitrogen-doped carbon supports (M-N-C) possess M-N_x sites that resemble those found in natural enzymes (Jiao et al., 2020; Wu et al. 2020b, 2020c). Recently, a laccase-like material Fe anchored N-doped carbon has been investigated to detect and degrade the phenolic pollutants (Lin et al., 2022; Sun et al., 2023). However, research on single-atom catalysts for simultaneous electrochemical quantitative detection and degradation of phenols is yet to be explored. Furthermore, the catalytic reaction mechanism occurring on the surface of nanozyme materials remains unclear.

Herein, enlightened by the structural features of active sites in natural laccases, we have designed and synthesized a single-atom copper nanozyme material with nitrogen-doped coordination modes. Specifically, the Cu²⁺-N tetrahedral coordination mode effectively mimics the coordination environment between Cu²⁺ and the imidazole group of amino acid side chains in the active site region of natural laccases. The morphology and structure of the CuN4-G nanomaterials were characterized and the microscale reaction kinetics and mechanism of guaiacol oxidation on the CuN₄-G surface were investigated using density functional theory (DFT) calculations. The oxidation process of guaiacol resulted in the appearance of characteristic peaks in cyclic voltammetry (CV) and differential pulse voltammetry (DPV), as depicted in Scheme 1. In practical applications, we utilized the developed electrochemical sensor to analyze orange juice samples contaminated with A. acidoterrestris at various storage times. The results demonstrated that this sensor has the potential to be applied in evaluating food quality. Furthermore, we assessed the laccase-like activity, stability, and degradation efficiency of CuN₄-G. The results indicated that CuN₄-G holds great promise for electrochemical detection and degradation of guaiacol.

2. Experimental

2.1. Materials synthesis

Graphene oxide (GO) was synthesized by a modified Hummer method (Hummers and Offeman, 1958). 100 mg of GO and 1.9 mg of CuCl₂·2H₂O (Cu/GO = 0.7%) were dissolved into 50 mL of distilled water. After sonicating for 1 h at 25 °C, a homogeneous solution was formed. Subsequently, the solution was treated to remove water in the system. Then, the pre-product was calcined at 750 °C in NH₃ and Ar atmospheres for 2 h to obtain CuN₄-G and CuG, respectively. Additionally, Cu-NG products were synthesized at different annealing temperatures, and the resulting products were named Cu-NG-450°C, Cu-NG-550°C, Cu-NG-650°C, and CuN₄-G. GO was calcinated with 750 °C for 2 h in Ar and NH₃ atmosphere to prepare graphene (G) and nitrogen-doped graphene (NG), respectively.

2.2. Evaluation of laccase-mimicking activities

The catalytic activity of CuN₄-G was examined by oxidation of 2,4-DP. 100 μ L 4-AP (10 mM) and 100 μ L 2,4-DP (10 mM) were combined



Fig. 1. The structural characterization of CuN_4 -G nanozyme. (a) Representative TEM image and (b) HAADF-STEM image of CuN_4 -G. (c) Representative high-resolution STEM image of CuN_4 -G and (d) STEM-EDS elemental mapping. (e) High-resolution XPS Cu 2p of CuN_4 -G. XPS spectrum (f) and high resolution of C1s (g) and N1s (h) spectra of CuN_4 -G. (i) Normalized XANES spectra at Cu K-edge of Cu foil, Cu_2O , CuO, CuN_4 -G. (j) FT-EXAFS spectra of CuN_4 -G at the Cu K-edge. (k) Atomic model of CuN_4 -G obtained from DFT calculation.

with 700 μ L MES buffer (60 mM, pH = 6.2). Next, 100 μ L CuN₄-G or laccase (1 mg mL⁻¹) was added to the aforementioned mixture. The system was reacted for 1 h at room temperature. Following the above reaction, the obtained supernatant was performed centrifugation. To acquire steady-state kinetic and catalytic performance of CuN₄-G, different concentrations of 2,4-DP (0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mM), 4-AP (1.5 mM), and 0.1 mg mL⁻¹ either CuN₄-G nanozyme

or laccase were combined, and UV–Vis analysis was performed on the resulting system at a wavelength of 510 nm. Michaelis-Menten equation (1) was used to calculate the Michaelis-Menten constant (K_m) and the maximum reaction velocity (V_{max}).

$$V_0 = (V_{\max} \times [S])/(K_m + [S])$$
(1)

where V_0 is the initial catalytic velocity and [S] is the substrate



Fig. 2. (a) UV–vis spectra of four reaction systems. (b) Lineweaver-Burk plots of the CuN₄-G nanozyme and laccase. (c) Michaelis-Menten curves for CuN₄-G nanozyme and laccase. (d) Reaction of 2,4-DP and 4-AP catalyzed by CuN₄-G. (e) The comparison of catalytic activity between the CuN₄-G nanozyme and laccase.

concentration (2,4-DP).

The mass concentration-related catalytic efficiency (k_{cat}/K_m) was derived by Eq. (2):

$$k_{\rm cat}/K_{\rm m} = V_{\rm max}/[E_0] \cdot K_{\rm m} \tag{2}$$

where k_{cat} is the catalytic constant and $[E_0]$ is the initial mass concentration of the catalyst.

2.3. Determination of real samples

Four types of fruit juices, namely orange juice, peach juice, apple juice, and a compound juice from three different brands, were selected to determine the guaiacol levels. This assessment aimed to evaluate the practicality of CuN₄-G-based electrochemical sensors. To prepare the samples for testing, all juice samples were diluted with PBS buffer at a specific pH. Subsequently, DPV response curves were recorded for the spiked samples using the standard addition method.

For investigating the impact of bacterial activity on guaiacol levels, sterile fruit juice without any preservatives was inoculated with *A. acidoterrestris* suspension. The *A. acidoterrestris* concentration was set at 0.5×10^5 CFU mL⁻¹, and 10 µg mL⁻¹ of vanillic acid was injected and then the system was incubated at 45 °C. Control samples were prepared without the addition of vanillic acid. The enumeration of bacteria in the juice samples was carried out at 0, 4, 8, and 12 h. HPLC and CuN₄-G sensor were used to the analysis of guaiacol in juice samples.

2.4. Catalytic degradation of guaiacol produced by A. acidoterrestris

Typically, 100 μ L of fruit juice infected with *A. acidoterrestris*, 100 μ L of CuN₄-G and 800 μ L of MES buffer were mixed. Then, the resulting mixture was conducted for various durations (1, 2, 6, and 8 h). After the specified reaction time, the CuN₄-G nanozyme was separated by centrifugation, and the guaiacol degradation efficiency of supernatant

was analyzed at the absorbance of 274 nm. For the control groups, deionized water was used instead of the CuN₄-G nanozyme under the same experimental conditions. Furthermore, under the same procedure, the mixed system was completed at various temperatures (from 40 to 80 °C) for various time intervals.

3. Results and discussion

3.1. Characterizations of CuN₄-G nanozyme

The catalyst morphology was characterized using TEM. CuN_4 -G presented a multilayer appearance with some fold, and no obvious aggregation of Cu nanoparticles was observed (Fig. 1a). Graphene possesses a large surface area and a layered structure, which might reduce the possibility of particle agglomeration so as to ensure the uniform dispersion of Cu species. HAADF-STEM image (Fig. 1b) shows that a large number of Cu atoms are evenly distributed in an independent form on graphene rather than in clusters. In the element mapping analysis of CuN_4 -G (Fig. 1c and d), the uniform distribution of Cu, O, N, and C in the architecture is obviously observed.

The oxidation state of Cu ions in Cu-NG was determined to be Cu²⁺ according to the binding energy of Cu $2p_{3/2}$ and Cu $2p_{1/2}$ centered at 933.3 and 955.3 eV, respectively, as shown in the X-ray photoemission spectroscopy (XPS) spectra in Fig. 1e. Besides, a satellite peak was observed locating at 943.5 eV (Fig. 1e). The full XPS spectrum of CuN₄-G exhibits peaks corresponding to Cu, O, N and C elements (Fig. 1f–h). The C 1s spectrum reveals three characteristic peaks at 284.6 (for C=C), 285.5 (for C–N/C–C), and 286.2 eV (for C–O) (Guan et al., 2020). Based on the high-resolution analysis of N 1s spectrum, N 1s spectrum is resolved into five peaks, centered at 397.9 (for pyridinic N), 398.5 (for Cu–N), 399.5 (for pyrrolic N), 400.8 (for graphitic N), and 402 eV (oxidized N) (Lai et al., 2017; Ni et al., 2018). Notably, characteristic peak of N 1s is observed in the CuN₄-G material, indicating that N was

successfully embedded into the carbon matrix.

To further determine the coordination environment and valence state of Cu in CuN₄-G, X-ray absorption near-edge structure (XANES) and X-ray absorption fine structure (EXAFS) were carried out. XANES profile of CuN₄-G is situated between those of Cu₂O and CuO, indicating that the valence state of Cu in CuN₄-G is between +1 and + 2 (Fig. 1i). In addition, the structure of CuN₄-G was disclosed by EXAFS. As shown in Fig. 1j, CuN₄-G exhibits a distinct peak at 1.78 Å, corresponding to the Cu–N bond and the coordination number is four. This result provides evidence for the CuN₄-G configuration (1.79 Å) as shown in Fig. 1k. Furthermore, no significant Cu–Cu peak is observed at 2.52 Å for CuN₄-G, confirming that Cu ions are atomically dispersed.

From the FT-IR analysis (Fig. S1) of CuN₄-G and Cu-G, significant shifts in peak positions can be observed. Specifically, there are notable changes in the peak positions corresponding to the C–N bonding in the range of 1000–1690 cm⁻¹, as well as the bonding between carbon and nitrogen in the range of 3100–3500 cm⁻¹, which are associated with the presence of pyridine and pyrrole functional groups. The Raman spectrum of CuN₄-G (Fig. S2) exhibits two representative peaks at 1352 (defective band) and 1600 cm⁻¹ (graphitic band), which reflect the level of defect in the material. After calculation, the peak intensity of the I_D/I_G of CuN₄-G (1.0) is found to be greater than that of Cu-G (0.86), suggesting that N doping produced more edge and structural defects in the graphene and increased its level of disorder (Guo et al., 2016; Jia et al.,



Fig. 3. (a) The intermediate structures of surface O species on the CuN_4 -G. (b) The free energy diagram of 2,4-DP oxidation reaction pathways on the surface of four catalytic models. (c) The rate-determining step barrier vs the difference between the adsorption free energy of *O and *OH for the four nanozyme models. The projected DOS for the CuN_4 -G nanozyme (d) and the charge density differences of CuN_4 -G (e and f). The red and blue areas represent the accumulation and depletion of charge density in the 3D map, respectively.

2016). Based on the subsequent electrochemical performance test results, it is further confirmed that N doping can promote the catalytic performance.

3.2. Mimicking laccase activity of CuN₄-G nanozyme

2,4-DP was chosen as a chromogenic substrate to assess the laccaselike activity of CuN₄-G. 2,4-DP is oxidized by CuN₄-G nanozyme and further reacts with 4-AP to form the red antipyridinone imide dye, which correspondingly shows a distinct absorption peak at 510 nm (Fig. 2a) (Wang et al., 2014). To investigate the actual origin of the catalytic reaction, a control experiment was conducted (Fig. S3). CuN₄-G was dispersed in water and centrifuged to obtain supernatant and precipitate. Negligible color change was observed in the supernatant with the introduction of 2,4-DP and 4-AP, indicating its lack of catalytic activity. However, the reaction solution turned red after introducing the precipitate, demonstrating that the laccase-like activity is originated from CuN₄-G nanozyme.

Some enzymes, such as glucose oxidase, are capable of oxidizing the substrate through the reaction of O_2 to H_2O_2 . On the other hand, laccase, acting as a green catalyst, can directly reduce O_2 to H_2O . In order to determine the reduction products of oxygen, a mixture of 2,4-DP and CuN₄-G was subjected to centrifugation after undergoing a reaction at 25 °C for 1 h (Fig. S4). As observed, the addition of saturated KI solution caused no significant change in color of the supernatant. Subsequently, H_2O_2 was added to the mixture, leading to an immediate yellow coloration. This outcome indicates that the $O_2 \rightarrow H_2O$ for CuN₄-G, rather than $O_2 \rightarrow H_2O_2$. Consequently, CuN₄-G exhibits laccase-like activity.

The steady-state kinetics were determined using various concentrations of 2,4-DP to better study the catalytic performance of CuN₄-G and laccase (Fig. 2b and c). The K_m and V_{max} for CuN₄-G and laccase were estimated by the Michaelis-Menten equation. The catalytic mechanism of 2,4-DP and 4-AP is illustrated in Fig. 2d (Wang et al. 2019b, 2022). The oxidation of 2,4-DP undergoes on the surface of CuN₄-G, while 4-AP reacts with the resulting oxidation product to generate a colored product. The V_{max} of CuN₄-G nanozyme (22.12 × 10⁻⁵ mM s⁻¹) is 4.7 times higher than that of laccase (4.67 × 10⁻⁵ mM s⁻¹), indicating a higher reaction rate (Fig. 2e). Furthermore, k_{cat}/K_m of CuN₄-G nanozyme is determined to be 442.4 × 10⁻⁵ (g L⁻¹)⁻¹ s⁻¹, surpassing that of laccase (Table S1). Compared to other previously reported laccase-like nanozyme materials (Table S1), CuN₄-G demonstrates significantly higher V_{max} and k_{cat}/K_m , underscoring the superior catalytic activity.

The stability of enzymes is of great importance in practical applications (Zhang et al., 2015). The catalytic stability of CuN₄-G and laccase under different pH, temperatures, ionic strength, and ethanol volume ratios was investigated. CuN₄-G exhibits excellent thermal stability with temperatures ranged from 30 °C to 90 °C, maintaining 95% initial activity even at 90 °C for 30 min (Fig. S5a). In contrast, laccase activity decreases significantly after 60 °C and is almost inactivated over 90 °C. Fig. S5b shows the impact of various pH values on the laccase-like activity. Only 5.5%–15.2% of laccase activity is remained within the pH range of 3–5. The catalytic activity grew initially before decreasing as the pH was raised, nearly disappearing at pH = 8. In the case of CuN₄-G nanozyme, 65%–94% of initial catalytic performance is remained within the pH range of 6–9. These results demonstrate that CuN₄-G exhibits superior performance throughout a broad pH range.

As shown in Fig. S5c, with the concentration of NaCl increasing, laccase performance shows an initial rise followed by a fall. Interestingly, the catalytic activity of CuN_4 -G increases. This may be attributed to the oxidation of -Cl by O₂ to reactive chloride species (RCSs), which further oxidizes 2,4-DP (Shan et al., 2016). Furthermore, high concentrations of NaCl reduce the solubility of the substrate due to the salt-out effect, promoting the adorption of 2,4-DP on CuN₄-G and enhancing its catalytic activity (Liang et al., 2017; Wang et al., 2019b).

Additionally, the influence of different ethanol concentrations on the catalytic activity was investigated. Fig. S5d illustrates the catalytic

activity of laccase decreases with increasing ethanol concentration (from 0 to 50% (v/v)). Conversely, the catalytic activity of CuN₄-G reaches 132% at 10% (v/v) ethanol concentration, which can be ascribed to the fact that a certain quantity of alcohol can enhance the solubility and dispersion of the reactants (Lin et al., 2022). In the meantime, the recyclability of CuN₄-G nanozyme was examined, as shown in Fig. S5e. The CuN₄-G nanozyme maintains 80% of its catalytic activity even after eight cycles. In comparison to homogeneous laccase, which is challenging to recycle, CuN₄-G nanozyme exhibits exceptional recyclability and stability under diverse conditions. The activity of laccase in water is significantly decreased. However, CuN₄-G still maintains 63% catalytic activity after 20 days at room temperature (Fig. S5f), suggesting that CuN₄-G has high stability in water.

3.3. Mechanism study of laccase-like activity of CuN₄-G by DFT calculations

To further investigate the catalytic activity mechanism of CuN₄-G nanozyme, the substrate's reaction process on the surface of the CuN₄-G nanozyme was analyzed by DFT calculations. We constructed three other nanozyme models with varying levels of laccase activity, namely CH–Cu (Wang et al., 2019b), CA-Cu (Wang et al., 2022), and Bpy-Cu (Li et al., 2022) for comparative purposes. In the reaction system, 2,4-DP is utilized as the catalytic substrate and is oxidized by surface oxygen molecules on the nanozyme (2,4-DP + $O_2 \rightarrow 2$,4-DP_{ox} + H₂O), resulting in the formation of radical products. The catalytic reaction steps are as follows (equations (3)–(6)), where the asterisk (*) represents nanoenzyme materials:

$$* + 2,4-DP + O_2 \rightarrow *OOH + 2,4-DP_{ox}$$
(3)

$$*OOH + 2,4-DP \rightarrow *O + 2,4-DP_{ox} + H_2O$$
 (4)

$$*O + 2,4-DP \rightarrow *OH + 2,4-DP_{ox}$$
(5)

$$*OH + 2,4-DP \rightarrow * + 2,4-DP_{ox} + H_2O$$
 (6)

Firstly, the DFT calculation method was utilized to determine the Gibbs free energy values of three crucial reaction intermediates, on the surface of CuN_4 -G nanozyme, namely *OOH, *O, and *OH. The optimized structures of these surface reaction intermediates on CuN_4 -G are depicted in Fig. 3a. Furthermore, Fig. 3b illustrates the reaction pathways for the oxidation of 2,4-DP by surface oxygen molecules for the four catalyst models. Similarly, the corresponding structures of surface reaction intermediates for the CH–Cu, CA-Cu, and Bpy-Cu models were calculated and optimized, as illustrated in Figs. S6–S8.

In the first reaction step, the energy of the surface intermediates significantly decreases for all four models. The energy values for CuN₄-G, CH-Cu, CA-Cu, and Bpy-Cu are -4.57 eV, -4.21 eV, -4.98 eV, and -5.21 eV, respectively. This indicates that all four nanozyme materials effectively adsorb and activate oxygen molecules, resulting in the extraction of hydrogen from 2,4-DP and the formation of *OOH intermediates. Subsequently, in the following steps, *OOH extracts hydrogen from the second 2,4-DP molecule, while the O-O bond breaks and dissociates from the material surface, generating a water molecule and *O intermediate. During this process, the energy of *O intermediates on the surface of CA-Cu and Bpy-Cu models further decreases without any significant barriers. However, both CuN₄-G and CH-Cu models exhibit noticeable barriers of 0.59 eV and 1.44 eV, respectively. This indicates that the formation of *O intermediates on the surfaces of CuN₄-G and CH-Cu encounters a barrier, which becomes the rate-determining step of the overall reaction process. In the third reaction step, hydrogen from 2,4-DP molecule can be easily extracted by *O intermediates on the surfaces of CuN4-G, CH-Cu, and CA-Cu, leading to the formation of *OH intermediates without any significant barriers. However, on the Bpy-Cu surface, *O intermediates need to overcome a high barrier of 2.34 eV to generate *OH intermediates. Thus, this step



Fig. 4. (a) Cyclic voltammogram of CuN_4 -G modified electrode at different scan rates from 10 to 200 mV s⁻¹. (b) The linear relationship of the redox peak currents on the scan rates. (c) DPV response of CuN_4 -G modified electrode for different guaiacol concentrations and (d) Corresponding calibration plot. (e) EIS responses of bare GCE, G/GCE, NG/GCE, CuG/GCE and CuN_4 -G/GCE in 5 mM [Fe(CN)₆]^{3-/4-} solution. (f) The oxidation process of guaiacol on the electrode surface.

becomes the rate-determining step for the reaction on the Bpy-Cu surface. In the final reaction process, the reaction on the CA-Cu surface exhibits typical endothermic characteristics, as water molecules need to absorb 1.83 eV of energy to dissociate from the material surface and complete the entire catalytic reaction cycle. This step becomes the ratedetermining step for the reaction on the CA-Cu surface. On the other hand, no significant barriers are observed for the other three nanozyme surfaces. Therefore, the above results indicate that in the entire catalytic reaction process, the nanozyme materials CH–Cu, CA-Cu, and Bpy-Cu exhibit higher barriers in their rate-determining steps (1.44 eV, 1.83 eV, and 2.34 eV, respectively) than CuN₄-G (only 0.59 eV), as shown in Fig. 3b and c. Hence, CuN₄-G demonstrates higher laccase-like activity, which is consistent with the experimental findings (Table S1).

According to the DFT calculations, the projected density of states (DOS) for CuN_4 -G were obtained and depicted in Fig. 3d. The projected DOS results reveal that, in comparison to the other three materials, the CuN_4 -G nanozyme exhibits a favorable alignment of the conduction and valence band (Fig. S9). This feature facilitates the easier transfer of photoexcited charge carriers and promotes the catalytic reaction process. Moreover, we explored the charge density of the four nanozyme models. As shown in Fig. 3e and f, the electron density of Cu atoms at the active site in the CuN_4 -G nanozyme is significantly increased compared

to other materials, owing to the bonding electron coupling effect facilitated by the structural characteristics of single-atom metal-nitrogen doping (Fig. S10). In conclusion, the single-atom metal-nitrogen doping contributes to the higher catalytic activity of the CuN₄-G nanozyme, which is consistent with the aforementioned results.

3.4. Electrochemical application of CuN_4 -G nanozyme in guaiacol detection

The electrocatalytic performance of the CuN₄-G-based electrochemical sensor was conducted using CV and DPV curves. As depicted in Figs. S11a and b, the electrochemical signal is closely related to the loading of Cu and N. The order of the oxidation current values is as follows: $G < CuG < NG < CuN_4$ -G. Furthermore, Fig. S11c illustrates the current response of Cu-NG samples obtained at varying annealing temperatures. Evidently, the annealing temperature has a vital impact on the performance. At an annealing temperature of 750 °C, CuN₄-G exhibits the highest electron transfer ability. Similar trend can be observed in the DPV curves (Fig. S11d).

Fig. S12 illustrates the electrocatalytic performance of different modified electrodes toward guaiacol oxidation. In comparison to G and CuG, NG exhibits a noticeable increase in the oxidation current,

Table 1

Comparison of the performances of CuN₄-G with other nanomaterials.

Electrode	Linear range (μ mol L $^{-1}$)	LOD (µmol L ⁻¹)	Ref.
Pt-γ-Al ₂ O ₃ /GCE Reduced graphene oxide/ GCE	0.05–30 0.5–500	1.79×10 ⁻² 0.2	(Sun et al., 2015) (Wu et al., 2014)
Lac ^a /CuNP's/CHIT ^b / cMWCNT ^c /PANI ^d /Au electrode	1–500	0.156	(Chawla et al., 2011)
Lac/MnO ₂ NPs/cMWCNT/ PANI/Au electrode	0.1–10 and 10-500	4×10^{-2}	(Rawal et al., 2012)
MFG ^e /GCE	0.03–30	1.3×10^{-3}	(Gan et al., 2014)
PEI ^f -AuNP-Lac/GCE	0.79-17.42	3×10^{-2}	(Brondani et al., 2013)
GNF ^g /GCE	0.03–46	9.8×10^{-3}	(Kalaiyarasi et al., 2017)
SG-GN ^h /GCE CuN ₄ -G/GCE	$\begin{array}{l} 0.0212 \\ 5 \times 10^{-6}5 \times \\ 10^{-2} \end{array}$	${\begin{array}{*{20}c} 5 \times 10^{-3} \\ 1.2 \times 10^{-6} \end{array}}$	(Li et al., 2019) This work

^a Lac: laccase.

^b CHIT: chitosan.

^c cMWCNT: carboxylated multiwalled carbon nanotube.

^d PANI: polyaniline.

^e MFG: MnO₂ nanoflowers graphene oxide.

^f PEI: polyethyleneimine.

^g GNF: graphene nanoflake.

^h G-GN: sodium gallate-exfoliated graphene nanosheets.

indicating that N doping enhances the structure and edge defects on graphene, thereby providing more active sites. Interestingly, the electrode modified with Cu and N co-doped graphene exhibits the highest peak current. The enhanced catalytic activity of the CuN4-G-based sensor toward the electrooxidation of guaiacol indicates that the synergistic effect between Cu and N plays a vital role. The relationships between the peak current of guaiacol and the annealing temperatures were analyzed in 0.1 M PBS (pH = 7.0) with 1.0 nM guaiacol using DPV and CV (Fig. S13). The results demonstrate that the modified electrode exhibits the highest sensitivity to guaiacol when the annealing temperature was 750 °C. The CuN₄-G modified electrode has enhanced sensitivity because of the formation of Cu-N₄ active sites at 750 $^\circ$ C. The influence of electrolyte pH on the electrochemical responses of guaiacol was explored through CV (Fig. S14a) and DPV (Fig. S14b) in the presence of 1.0 nM guaiacol. Fig. S14b exhibits a similar trend, where the peak current reaches a maximum as the pH increases from 3 to 6. However, the current gradually decreases when the pH exceeds 6. The relationship between the response current and pH can be described by the linear equation: y = -0.0522x + 0.4826 ($R^2 > 0.99$) (Fig. S14c). Additionally, the electrochemical redox peak exhibits a negative shift, indicating the involvement of protons in the reaction of guaiacol on the electrode (Zhang et al., 2021).

To further investigate the redox kinetics of guaiacol on the CuN4-G nanozyme electrode, different scan rates were examined using CV for 1.0 nM guaiacol (Fig. 4a). As depicted in Fig. 4b, the oxidation current exhibits a linear increase with the escalating electrochemical scan rate, indicating a positive correlation between the oxidation current and the square root of the scan rate ranging $0.01-0.2 \text{ V s}^{-1}$. This observation suggests that catalytic reaction of guaiacol on the CuN₄-G modified electrode follows a diffusion-controlled process (Ulloa et al., 2021). The electrocatalytic response of CuN4-G to various concentrations of guaiacol was investigated using DPV (Fig. 4c). The peak current shows an increase linearly to the guaiacol concentration (5-50,000 pM). The regression linear equation between the response current and the guaiacol concentration is given as y = 14.24x + 43.8 ($R^2 = 0.994$), where y is the oxidation current and x is the logarithm of the target substrate (Fig. 4d). Furthermore, electrochemical impedance spectroscopy (EIS) (Fig. 4e) reveals the charge transfer resistance (R_{ct}) in the



Fig. 5. The repeatability (a), the anti-interference study (b) and the stability (c) of the CuN₄-G-based electrochemical sensor in 0.1 M PBS.

higher frequency region, while a linear cross-section is observed in the lower frequency region. The diameter of the EIS semicircle is allowed for the analysis of the electron transfer resistance (R_{et}) . Compared to the bare electrode, the CuN₄-G electrode exhibits decreased impedance with a lower R_{ct} value of 34.8 Ω , indicating that CuN₄-G promotes electron transfer. This result further validates the previous theoretical computational findings (Fig. 3e and f). This observation is consistent with the CV and DPV results, indicating that the CuN₄-G/GCE was successfully prepared. The guaiacol oxidation process is shown in Fig. 4f. Based on DFT, we calculated the energy transition value ($E_g = 0.215 \text{ eV}$) between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) of the guaiacol molecule. The limit of detection (LOD) was determined to be 1.2 pM (S/N = 3) for guaiacol. The prepared sensing platform demonstrates a lower detection limit compared to previous studies (Table 1), highlighting its excellent detection and analytical capabilities for guaiacol.

The repeatability of CuN_4 -G was assessed using DPV, where the same electrode was continuously tested 10 times at 5.0 nM guaiacol. The RSD of the peak current is less than 1%, as shown in Fig. 5a, demonstrating



Fig. 6. (a) The colony count concentration of *A. acidoterrestris* in orange juice. (b) Detection of guaiacol with HPLC. (c) The production of guaiacol by *A. acidoterrestris* in the presence of vanillic acid determined with CuN_4 -G. (d) Plot of the redox peak current and (e) HCA dendrogram *vs* storage times, respectively. (f) ROC curve of the electrochemical sensor in the detection of guaiacol. (g) The degradation efficiency of CuN_4 -G for guaiacol at different temperatures and reaction time. (h) Schematics of guaiacol identification of the orange juice based on the electrochemical sensor.

that the CuN₄-G-based sensor exhibits excellent repeatability. In addition, the anti-interference ability of the sensor was assessed by introducing various common interfering substances, including 0.1 M Cu²⁺, Fe³⁺, Ca²⁺, Cl⁻, SO₄²⁻, glucose (G), citric acid (CA), ascorbic acid (AA), vanillin (VAN), uric acid (UA), and guaiacol, into water samples (Fig. 5b). It was observed that the current response remained significant when 5.0 nM guaiacol was added to PBS. However, after the addition of a series of interfering substances and their mixtures, the current changes were negligible, indicating that the proposed sensing method exhibits high selectivity for detecting guaiacol. To assess the stability of the proposed sensor, the current responses of guaiacol were monitored after storing the same electrode at 4 °C. Electrochemical analysis of guaiacol on CuN₄-G was conducted at intervals of 10 d. After 20 d, the oxidation peak current retained 84.79% of its initial response, indicating satisfactory stability and long-term storage capability of CuN₄-G (Fig. 5c).

3.5. Detection of guaiacol in fruit juice infected with A. acidoterrestris

A. acidoterrestris has the ability to product guaiacol from the precursor vanillic acid in fruit juices. When the guaiacol content exceeds 2 μ g L⁻¹, it imparts a distinct medicinal or antiseptic off-odor. In order to assess the potential application of the prepared CuN₄-G sensor in real food systems, the following experiments were conducted. In this study, orange juice samples infected with *A. acidoterrestris* were analyzed using both HPLC and the proposed electrochemical sensor. As the storage time increases, the colony count concentration of A. acidoterrestris in orange juice gradually increases, as shown in Fig. 6a. In the experimental group, the peak area gradually increases from 0 to 12 h of culture time, as shown in Fig. 6b. This increase can be ascribed to the conversion of vanillic acid by A. acidoterrestris to guaiacol. Furthermore, the characteristic peak value of oxidation current gradually increases with time, as depicted in Fig. 6c, showing a consistent trend with the HPLC results. Moreover, a significant difference in the violin chart score of the redox peak current vs storage times was observed (Fig. 6d). The hierarchical cluster analysis (HCA) was performed to visually illustrate the classification capacity of the proposed electrochemical sensor in a more intuitive manner. The raw training data was subjected to HCA using the Euclidean distance metric. Euclidean distance is a common distance metric used to calculate the similarity or distance between data points. Fig. 6e clearly demonstrates accurate and distinct distribution of all four types of orange juice into their respective groups, without any misclassifications. Furthermore, the orange juice samples collected after 8 and 12 h of storage were grouped together, as they exhibited a similar, elevated guaiacol concentration when compared to the samples obtained at 0 h. This observation highlights a consistent similarity in guaiacol levels between the orange juice samples stored for 8 and 12 h, justifying their clustering in the HCA analysis. The receiver operating characteristic (ROC) curve was employed to evaluate the performance of

the electrochemical sensor for guaiacol identification. Fig. 6f illustrates that the area under the curve (AUC), true positive rate (TPR), and false positive rate (FPR) of this food classification model are 1, 1, and 0, respectively, indicating that this electrochemical sensor has high accuracy to detect guaiacol produced by A. acidoterrestris.

Additionally, the feasibility and suitability of this sensor were verified in three other types of juice samples, including peach juice, apple juice, and compound juice. The concentrations of guaiacol are determined and presented in Table S2. The recovery rate ranges from 94% to 106%, demonstrating the high reliability and potential application of the CuN₄-G-based sensor for guaiacol detection in real samples. All results acquired using the CuN₄-G sensor are in line with those obtained using the HPLC approach, demonstrating acceptable accuracy of the developed strategy compared to HPLC results.

Guaiacol present in fruit juice negatively affects its flavor. Therefore, the removal of guaiacol from fruit juice is necessary. The degradation efficiency of guaiacol by CuN₄-G nanozyme was evaluated. As depicted in Fig. 6g, the efficiency of CuN₄-G in degrading guaiacol increases with prolonging reaction time. Furthermore, the influences of different temperatures on guaiacol degradation were studied. As the temperature increases from 40 °C to 60 °C, the degradation rate also reinforces. At 60 °C, the degradation rate reaches 81.13% after 8 h. This demonstrates that the CuN₄-G nanozyme is appropriate for practical applications, and the issue of high-temperature-induced enzyme inactivation can be disregarded. The principle of the proposed sensor for the analysis of guaiacol, based on CuN4-G nanozyme as the sensing electrode, is illustrated in Fig. 6h.

4. Conclusion

In summary, we have successfully synthesized single-atom copper anchored N-doped graphene (CuN₄-G) with the potentiality of efficient degradation and sensitive detection of guaiacol. The CuN₄-G nanozyme exhibited excellent laccase-like activity for guaiacol detection, owing to the synergistic effect of the single-atom metal-nitrogen doping structure. DFT calculations demonstrated that CuN4-G facilitated the electron transfer and enhanced the catalytic reaction process. The CuN₄-G-based sensor displayed a wide linear range of 5-50,000 pM and a low detection limit of 1.2 pM. Furthermore, the developed sensor exhibited outstanding anti-interference properties and stability, and was successfully applied for detecting and degrading of guaiacol produced by A. acidoterrestris in fruit juice. This study not only gives us new hints to the design and preparation of bifunctional single-atom nanozymes but also offers a novel strategy for detection and degradation of phenolic substrates.

CRediT authorship contribution statement

Xiaodi Niu: Writing - original draft, Methodology, Investigation, Data curation, Software. Lifang Wu: Writing - original draft, Methodology, Investigation, Data curation, Software. Fengling Wu: Methodology, Investigation, Data curation, Software. Jingqi Guan: Methodology, Investigation, Data curation, Software. Hongsu Wang: Project design, Formal analysis, Writing - original draft, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi. org/10.1016/j.bios.2023.115606.

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