A novel nitrite biosensor based on the direct electrochemistry of horseradish peroxidase immobilized on porous Co₃O₄ nanosheets and reduced graphene oxide composite modified electrode

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Abstract: Porous Co₃O₄ hexagonal nanosheets (PCHNSs) with a well-defined morphology have been synthesized successfully via a hydrothermal process and followed by calcinations. Co₃O₄ nanosheets immobilizing horseradish peroxidase (HRP), together with reduced graphene oxide, have been used to modify glassy carbon electrode to fabricate a mediator-free biosensor. The morphology and structure of PCHNSs are studied by XRD, FESEM, and HRTEM techniques. Spectroscopic and electrochemical results reveal that Co₃O₄/rGO composite film is an excellent immobilization matrix with biocompatibility for enzymes, affording good enzymes' bioactivity and stability. Meanwhile, due to the unique structure of the modified electrode, the direct electron transfer of HRP is facilitated and the prepared nitrite biosensors display good performance---a wide linear range of 1 to 5400 μ M, an extremely low limit of detection (LOD) as 0.21 μ M and a limit of quantification as (LOQ) as 0.58 μ M. This work suggests that Co₃O₄/rGO composite film is a potential matrix for enzymes' immobilization and biosensors' preparation.

Key words: Biosensor; Porous Co₃O₄ hexagonal nanosheets; Direct electron transfer; Nitrite

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1. Introduction

Nitrite ions is a typical pollutant, the levels of nitrite ions can provide an index of organic pollution in natural or sewage water when correlated with other forms of nitrogen[1, 2]. When nitrite ions exposure to dangerously high levels in drinking water, it can cause a number of adverse health effects especially on infants and children [3]. Some medical issues such as esophageal or gastric cancer are believed to be associated with nitrite ions intake [4-6]. Therefore, the U.S. Environmental Protection Agency (EPA) defined the maximum contamination level (MCL) of nitrite ions in drinking water to be 1.0 mg N L^{-1} ($71.4 \text{ }\mu\text{M}$) [7]. Thus quantitative determination of nitrite is of great importance in environmental and industrial processes, public health and control quality of foods. Several techniques have been developed to nitrite determination including spectrophotometry [8], chromatography [9], chemiluminescence [10], spectrofluorimetry [11] and capillary electrophoresis [12]. Furthermore, as a new type sensor, plasmonic sensors may have great application potential in NaNO_2 detection [13-16]. However, these techniques also have disadvantages such as time consuming and complicated extraction processes with specific reagents, poor sensitivity and serious interference from some species. Therefore, developing sensitive, specific, simple and low cost methods for determination of nitrite is greatly important.

Compared to other methods, electrochemical techniques, especially hemo protein constructed direct electrochemistry biosensor, provide useful alternatives for nitrite detection due to their advantages such as simplicity, fast analysis, high sensitivity and selectivity, without using any specific or derivative reagents. However, due to the electroactive center deep burying in the hemo protein's structure and easy denaturation of hemo proteins on the bare electrode surface, it is difficult for enzymes to realize the direct electrochemistry process on the bare electrode [17, 18]. Recent researches show that a series

of nanomaterials with special the quantum size effect and surface effect can be used to immobilize enzymes, facilitate the direct electron transfer and fabricate mediator-free biosensors [18, 19]. Among them, metal oxide nonmaterials, such as ZnO [20], SnO₂ [21], TiO₂ [22], MnO₂ [17], have received increasing attention. However, to the best of our knowledge, porous Co₃O₄ hexagonal nanosheets with good biocompatibility for constructing direct electrochemical biosensor have rarely been reported [23].

Here, porous Co₃O₄ hexagonal nanosheets are synthesized by a simple hydrothermal process and followed by calcinations. Co₃O₄ nanosheets immobilizing horseradish peroxidase (HRP), as a kind of hemo proteins, together with graphene, have been used to modify glassy carbon electrode to fabricate a mediator-free biosensor. As shown in scheme 1, due to the synergetic effects of Co₃O₄ and graphene, the prepared biosensor possesses many advantages. First, Co₃O₄ nanosheets with excellent biocompatibility may provide a protective microenvironment for HRP to retain its enzymatic stability and activity. The porous structure of the Co₃O₄ nanosheets can greatly enhance the active surface area available for enzymes adsorption. Second, rGO with better mobility of charge carriers can become the outstanding media of efficient electrical communication between the enzyme and the electrode. Third, the substrate can simply be entrapped by graphene with a high surface area, resulting substrate gets close to a large quantity of enzymes immobilised in the porous Co₃O₄ nanosheets. Concentration of substrate and enzyme in this confined region will increase the chance of effective collisions between them [24]. Therefore Co₃O₄/rGO composite film is a potential matrix for enzymes immobilization and biosensors preparation. In this work, the performance of a sensor composed of HRP immobilized in the Co₃O₄/rGO composite film is compared with that of sensors based on Co₃O₄ and rGO in order to highlight the advantages of the Co₃O₄/rGO composite film as a support.

2. Experimental

2.1. Materials and apparatus

HRP and Nafion solution (5 wt% in lower aliphatic alcohols) are purchased from Sigma. Unless otherwise noted, all other chemicals (analytical grade) are purchased from Sinopharm Chemical Reagent Co., Ltd., China. In all experiments deionized water is used.

X-ray diffraction (XRD) patterns are recorded on a Rigaku D/max 2200pc diffractometer using Cu K α radiation of wavelength $\lambda = 0.15418$ nm at 40 kV and 40 mA. Field-emission scanning electron microscopy (FE-SEM) is performed using a Hitachi S-4800 & Hiroba energy dispersive X-ray electron microscope. Transmission electronic microscopy (TEM) images are obtained on a FEI Tecnai F20 instrument. TG–DSC investigation is carried out on a Netzsch Corporation STA409PC). Zeta potential is performed by a Malvern Nano-Z system. UV-vis spectra are recorded using a PerkinElmer Lambda-950 spectrophotometer. All electrochemical experiments are performed with a CHI 660D electrochemical workstation (CH Instruments, Shanghai, China). A conventional three-electrode system which consists of a platinum wire as the counter electrode, an Ag/AgCl/3M KCl as the reference electrode and a 3-mm diameter modified glassy carbon electrode (GCE) as the working electrode is used in all electrochemical experiments. Unless otherwise noted, 0.1 M phosphate buffered saline (PBS; pH 7.0) is used as the supporting electrolyte in all experiments. The buffer is purged with highly purified nitrogen for at least 30 min and a nitrogen atmosphere environment is kept in electrochemical measurements.

2.2 Synthesis of porous Co₃O₄ hexagonal nanosheets

In a typical experimental procedures, 0.58 g of Co(NO₃)₂·6H₂O and 0.5 g of poly(vinylpyrrolidone) (PVP) were dissolved in a 20 mL mixture of ethanol and distilled water with a 1:1 volume ratio. After

30 min magnetic stirring, 20 mL of a 0.4 M NaOH aqueous solution was slowly added the mixture under continuous stirring, accompanying with the colour evolution from red to blue (Fig. S1). Then the reaction suspension was quickly transferred into a Teflon-lined autoclave. The autoclave was heated to 200 °C and maintained at this temperature for 12 h. After naturally cool to room temperature, the pink product (Fig. S2A) was collected by centrifugation and washed thoroughly with distilled water several times. The β -Co(OH)₂ precursors were obtained after drying for 12 h at 60 °C in air. The final product black Co₃O₄ (Fig. S2B) was prepared by annealing β -Co(OH)₂ at 300 °C for 2 h in air with a slow heating rate (1 °C/min).

2.3 Preparation of reduced graphene oxide

Graphene oxide (GO) was prepared from graphite powder by the modified Hummers method [25]. The reduced graphene oxide (rGO) was synthesized by a simple hydrothermal method. Fig. S3 gives the SEM image of GO and rGO, as well as Raman spectra of GO (a) and rGO (b).

2.4 Preparation of enzyme electrode

To investigate the electrochemical behavior of Co₃O₄-HRP/rGO/GCE, enzyme electrode was assembled. Prior to use, the GCE was sequentially polished using 1.0, 0.3 and 0.05 μ m alumina powder on a polishing cloth and rinsing with deionised water. The electrode was then sonicated in ethanol and deionised water. Next, the electrode was dried using a purified nitrogen stream. The enzyme electrode was prepared by a simple casting method. Firstly, 1 mL of 2 mg mL⁻¹ porous Co₃O₄ nanosheets suspension and 0.5 mL of 10 mg mL⁻¹ HRP PBS were mixed and then stirred for 30 min. Next, 0.5 mL of rGO (5%) was added to the mixture and then stirred for 10 min. Finally, 4 μ L of the mixture was dropped to the surface of a freshly polished GCE to prepare the Co₃O₄-HRP/rGO/GCE. A beaker was covered over the electrode so that water can evaporate slowly in air and a uniform film electrode can be

obtained. The dried Co_3O_4 -HRP/rGO/GCE was stored at 4°C in refrigerator when not use.

For comparison, Co_3O_4 nanosheets and rGO were used for the fabrication of Co_3O_4 -HRP/GCE and HRP/rGO/GCE through similar procedures as described above. In the control experiment, Nafion/HRP/GCE and Co_3O_4 /rGO/GCE were also prepared. Before electrochemical measurements, all the as-prepared film electrodes were immersed in pH 7.0 PBS for 30 min to remove residual components.

3. Results and discussion

3.1. Characterization of porous Co_3O_4 hexagonal nanosheets

X-ray diffraction (XRD) was firstly carried out to determine the structure of the as-prepared products. Fig. 1 presents the typical XRD patterns of the Co_3O_4 HNSs (b) and $\beta\text{-Co}(\text{OH})_2$ (a). As can be seen, all diffraction peaks of the as-synthesized pink precipitates can be indexed as a single phase of the hexagonal structured brucite-like $\beta\text{-Co}(\text{OH})_2$ (JCPDS No. 30-0443) [26]. After annealed at 300 °C, the precursors were thoroughly converted into pure cubic phase of Co_3O_4 (JCPDS No. 43-1003), and no other characteristic peaks are detected.

Fig. 2 shows typical SEM and TEM images of the samples prepared at various stages. From Fig. 2A, it is observed that $\beta\text{-Co}(\text{OH})_2$ are well-defined hexagonal sheets with the size in the range of 200-300 nm and the thickness in the range of 30-50 nm. Fig. 2B shows a magnified SEM image for representative hexagonal nanosheets, exhibiting regular hexagons with the angles of adjacent edges of 120° with smooth and clean surfaces. Fig. 2C exhibits the TEM images of synthesized $\beta\text{-Co}(\text{OH})_2$ hexagonal sheets, which shows full consistency with the observed results of SEM. The inset of Fig. 2C presents the high-resolution TEM (HRTEM) image. The spacing between adjacent lattice planes is 0.28 nm, corresponding to the (100) planes of hexagonal brucite-like $\beta\text{-Co}(\text{OH})_2$ [27]. Fig. 2D indicates that

calcinations at 300 °C for 120 min did not change the morphology of hexagonal sheets, which may be attributed to the mild calcination temperature and slow heating rate (1 °C/min). Meanwhile, based on the Fig. 2E and 2F, after thermal treatment, the surfaces of nanosheets became rough and porous, which may be attributed to the impact of gas evolution during the thermal decomposition reaction from β -Co(OH)₂. The inset of Fig. 2F presents the HRTEM image of the product after thermal treatment. The spacing between adjacent lattice planes is 0.46 nm, corresponding to the (220) planes of cubic Co₃O₄ phase.

Further, TG–DSC investigations are conducted to determine the thermal behavior of the as-obtained β -Co(OH)₂ shown in Fig. S4. The DSC plot presents one endothermic peaks at 206°C, resulting from the decomposition of β -Co(OH)₂ into Co₃O₄. From the TG plot, the relatively stable platform after 300 °C indicates that no further reaction related to the mass increased or decreased. The final weight loss is measured to be about 13.1%, which is in good agreement with the theoretical value (13.6%) changed from β -Co(OH)₂ to Co₃O₄.

3.2. Encapsulation of HRP in the Co₃O₄ /rGO composite film

Fig. S5 exhibits typical SEM image of Co₃O₄-HRP/rGO/GCE. From Fig. S5 A, a rough structure, increasing the electrode surface area greatly, can be found on the electrode. As can be seen from the magnification SEM images (Fig. S5 B and C), Co₃O₄ nanosheets and rGO are discernible. The Co₃O₄ nanosheets owning rough and porous structure can greatly enhance the active surface area available for adsorbing enzymes. In addition, due to the high specific surface area and the crumpled morphology of rGO, Co₃O₄ nanosheets are embedded in the rGO to form a stable composite film, which is essential for the stability of the prepared enzyme electrode.

Further, Zeta potential measurement shows that the prepared porous Co₃O₄ nanosheets carry

negative charge with a zeta potential of -12.8 mV. The isoelectric point (pI) of enzymes decides the surface electrical property of enzymes in solution. As we known, the pI of HRP ($pI = 8.9$ [28]) larger than pH 7.0. Hence, when pH (solution) ≤ 7.0 , the surface of HRP carries positive charge. Thus, positively charged HRP molecules in pH 7.0 PBS can be attached onto the Co_3O_4 nanosheets surface by the electrostatic interaction.

UV-vis spectroscopy is an effective method to monitor the structure change of heme proteins. The Soret absorption bands of heme proteins provide information about the conformational integrity of the proteins [29, 30]. As shown in Fig. 3, the Soret absorption band of HRP immobilized on $\text{Co}_3\text{O}_4/\text{rGO}/\text{GCE}$ (storage at 4°C for four weeks) is located at 405.3 nm (curve a), which is close to that of native HRP at 403.2 nm (curve b), indicating that HRP retained the essential features of its conformational integrity. This reveals that $\text{Co}_3\text{O}_4\text{-HRP}/\text{rGO}$ composite film has biocompatibility and does not suffer significant enzymes denaturation.

3.3. Direct electrochemistry behavior of $\text{Co}_3\text{O}_4\text{-HRP}/\text{rGO}/\text{GCE}$

Fig. 4 depicts the typical cyclic voltammograms (CVs) of different modified electrodes in 0.1 M PBS (pH 7.0) at a scan rate of 0.1 V s^{-1} . No redox peak is observed at $\text{Co}_3\text{O}_4/\text{rGO}/\text{GCE}$ (curve a), which indicates $\text{Co}_3\text{O}_4/\text{rGO}/\text{GCE}$ is electroinactive in the potential window. The $\text{Co}_3\text{O}_4\text{-HRP}/\text{rGO}/\text{GCE}$ (curve e) exhibits a couple of stable and well-defined redox peaks at -0.363 V and -0.326 V vs. Ag/AgCl , which can be ascribed to direct electron transfer between HRP and the underlying electrode. According to Xu et al. [31, 32], the electron transfer of HRP occurs between the Fe^{III} and Fe^{II} site inset HRP itself. The formal potential ($E^{0'} = (E_{\text{pa}} + E_{\text{pc}})/2$) of HRP is -0.344 V vs. Ag/AgCl is in agreement with -0.362 V, -0.369 V and -0.335 V vs. Ag/AgCl reported for the $\text{HRP}(\text{Fe}^{\text{III/II}})$ redox couple in HRP-graphene-Nafion, HRP-GO-Nafion and HRP-ZrP films [33-35]. The potential difference (ΔE_p)

between the anodic and cathodic peak potential is about 37 mV, slightly greater than the theoretical value of reversible surface attached reaction process (0 mV) [36], which reveals a quasi-reversible electron-transfer process. Compared with that of the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$, almost no redox peaks observed at the Nafion/HRP/GCE (curve b, in Fig. 4), suggesting an enhanced electron transfer between HRP molecules and the surface of the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$. Meanwhile, due to the deep burying of electroactive center in the enzymes structure and the denaturation of enzymes on the bare electrode surface, it is difficult for enzymes to realize the DET process on the bare electrode [37]. It can be clearly seen from the comparison that direct electron transfer between HRP molecules and GCE is greatly enhanced at the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$.

For comparison, $\text{Co}_3\text{O}_4\text{-HRP/GCE}$ and HRP/rGO/GCE have also been carried out (curve c and d, in Fig. 4). The redox peaks of the two electrodes are 46.3% (Co_3O_4) and 31.4% (rGO) smaller, respectively, than that of $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$. The ΔE_p are 69 mV ($\text{Co}_3\text{O}_4\text{-HRP/GC}$) and 55 mV (HRP/rGO/GCE) respectively which are larger than that of $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ (37mV), indicating slower electron transfer rate of HRP on the two electrodes than on $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$. It can be concluded that the unique structure of the modified electrode has better effect on the facilitation of electron transfer.

Fig. 5 shows the cyclic voltammograms of 0.1 M PBS (pH 7.0) at a $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ when the scan rate was increased from 0.05 to 0.5 V s^{-1} . With the increase of the scan rate, the cathodic and anodic peak currents of HRP increase simultaneously, meanwhile the cathodic and anodic peak potentials show a small shift and the peak to peak separation also become slightly enlarged. The inset of Fig. 5 shows that the cathodic (i_{pc}) and anodic (i_{pa}) peak currents increase linearly with scan rates from 0.05 to 0.5 V s^{-1} . This reveals that the electrode reaction corresponds to a surface attached reaction

process. Using the Laviron method for a surface-controlled electrochemical system [35], the apparent heterogeneous electron transfer rate constant (k_s) of HRP immobilized on Co₃O₄-HRP/rGO/GCE is estimated to be about 4.3 s⁻¹, which is higher than the value reported for HRP immobilized halloysite nanotubes/chitosan (3.31 s⁻¹) [38], β-cyclodextrin hybrid film (2.87 s⁻¹) [39] and flowerlike ZnO-gold nanoparticle-Nafion nanocomposite (1.94 s⁻¹) [40], suggesting a faster electron-transfer process. This means that the unique structure of Co₃O₄-HRP/rGO composite film facilitates the direct electron transfer of HRP.

The average surface concentration of electroactive HRP (Γ^* , mol cm⁻²) can be estimated from the charge integration of the reduction peak of the CV using the formula:

$$Q = nF\Gamma^* \quad (1)$$

where F^* is Faraday constant, Q is the charge (C) and can be obtained by integrating the reduction peak of HRP, n and A stand for the number of electron transferred and the geometrical surface area of the electrode, respectively. According to this method, the surface concentration of electroactive HRP (Γ^*) at Co₃O₄-HRP/rGO/GCE is calculated as 1.48×10⁻¹⁰ mol cm⁻², much greater than the theoretical value for monolayer coverage (1.89×10⁻¹¹ mol cm⁻²) [41]. This means that multilayers of HRP entrapped in the Co₃O₄-HRP/rGO/GCE participate in the electron-transfer process.

Fig. 6 shows the cyclic voltammograms of 0.1 M PBS of different pH at a Co₃O₄-HRP/rGO/GCE. Stable and well-defined CVs were observed in the pH range 6.0~8.0. With the increase of pH value, both the cathodic and anodic peaks shifted to the negative (Fig. 6). This can be contributed to the involvement of proton transfer in the HRP(Fe^{III})/HRP(Fe^{II}) redox couple [42]. The $E^{0'}$ value of HRP varied linearly in the range of pH 6.0~8.0, with a slop of -53.4 mV pH⁻¹ (the inset of Fig. 6). This value is very close to the theoretical value for the transfer of one proton and electron in a reversible reduction

(-58 mV pH⁻¹ at 25°C) [42, 43].

3.4. Electrocatalysis of Co₃O₄-HRP/rGO/GCE to reduction of nitrite

In order to study the electrocatalytic activity of HRP immobilized in Co₃O₄-HRP/rGO/GCE, its response to the reduction of nitrite was explored. Fig. 7A shows the CVs of Co₃O₄-HRP/rGO/GCE in 0.1 M PBS (pH 5.5) containing different concentrations of NaNO₂. Only a couple of stable and well-defined redox peaks at -0.293 V and -0.241 V vs. Ag/AgCl, which can be ascribed to direct electron transfer between HRP and the underlying electrode, is observed for Co₃O₄-HRP/rGO/GCE in PBS without NaNO₂. After adding NaNO₂, a new irreversible reduction peak at -0.710 V is observed, and the peak current increased linearly with the increase of NaNO₂ concentration. In contrast, no significant reduction peak of NaNO₂ is observed on Co₃O₄/rGO/GCE (the inset of Fig. 7A), which indicates that the irreversible reduction wave is not a result of the Co₃O₄ nanosheets or rGO.

It is worthwhile to notice that after adding nitrite into the buffer solution, redox peaks of HRP itself shift to negative potential (Fig. S6). According to the report of Bayachou et al. [44, 45], this phenomenon reveals that the HRP(Fe)-NO is formed on the surface of electrode and NO binds at the redox-active Fe site. The reduction product at near -0.710 V is most likely N₂O, and the mechanism of the electrocatalytic reduction of nitrite at HRP-based electrode can be proposed as follows [42, 43, 46]:

Obviously, the acidity of the detection solution has significant effect on the catalytic reduction of NO_2^- because the catalysis of HRP to NO_2^- is accompanied by the protons transfer. Therefore, the effect of the buffer solution pH value on the biosensor response in the presence of $400\ \mu\text{M}$ NaNO_2 is studied (Fig. 7B). Both the peak potential and current are depended on pH value. With the decrease of pH value, the peak potential shifted positively, suggesting a proton-coupled reaction [46, 47]. It is clearly seen from Fig. 7B that the peak current increased with the value of pH from 7.0 to 5.5. This may be attributed that the increasing concentration of H^+ as a reactant can facilitate electrocatalytic reduction of nitrite at HRP-based electrode (eq. 3-8). The peak current drops down from pH 5.5 to 4.0, resulting from the denaturation of HRP existed to a considerable extent when the pH shifted toward a more acidic direction. It is obviously that this biosensor has the optimum sensitivity at pH 5.5 for the detection of nitrite.

3.5. Biosensor performance of the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$

As is well known, nitrite is a toxicant which widely exists in polluted water and antiseptic food. It is vitally important to detect nitrite in the fields of biological and environmental chemistry. The biosensor performance for the detection of nitrite of the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ is also investigated by amperometry. The optimal applied potential has been confirmed prior by experiment due to its strong effect on the biosensor response. The applied potential is changed from $-0.5\ \text{V}$ to $-0.8\ \text{V}$ and the corresponding response current to $100\ \mu\text{M}$ NaNO_2 is measured (shown in Fig. S7A). As shown in From Fig.S7B, the response current increases rapidly as the applied potential changes from $0.5\ \text{V}$ to $0.7\ \text{V}$. This indicates that the response of the enzyme electrode is controlled by the electrochemical reduction of nitrite. When the potential is lower than $-0.7\ \text{V}$, a current plateau appears. The appearance of such a current plateau is attributed to the rate-limiting process of enzyme kinetics [48, 49]. In this study,

selecting -0.7 V as applied potential to detect NaNO_2 can make interfering reactions of other possible electroactive species in solutions minimize as well as the background current and noise levels lower relatively.

Fig. 8 gives the amperometric responses of $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ at -0.70 V after adding NaNO_2 in stirred PBS (0.1 M, pH 5.5). Fig. 8 demonstrates the response time (achieving 95% of the steady-state current) is less than 3 s. Moreover, a well-defined linear relationship between the current and the NaNO_2 concentration is observed. The calibration plot (Inset of Fig. 8) shows a linear range from 1 to 5400 μM . The linear regression equation is $I/\mu\text{A} = 0.293 (\pm 0.011) + 1.67 (\pm 0.011) \times 10^{-3} C/\mu\text{M}$ with a correlation coefficient of 0.993 (N=33). The limit of detection (LOD) is estimated to be 0.21 μM and limit of quantification (LOQ) is 0.58 μM . Performance of various nitrite biosensors is listed in Table S1. From Table S1, it is clear that, compared with other electrode, $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ not only has lower detection limit but also maintains a relatively wider linear range. Furthermore, compared with other prepared modified electrodes in this study (HRP/rGO/GCE (b), $\text{Co}_3\text{O}_4\text{-HRP/GCE}$ (c), Nafion/HRP/GCE (d) and $\text{Co}_3\text{O}_4\text{/rGO/GCE}$ (e)), $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ has the better performances (Fig. S8 and Table S2). First, porous Co_3O_4 nanosheets can greatly enhance the active surface area available for immobilizing HRP and also retain HRP's enzymatic stability and activity. More importantly, the substrate can simply be entrapped by rGO with a high surface area, permitting the access of a large quantity of enzymes immobilized in the porous Co_3O_4 nanosheets. Concentration of substrate and enzyme in this confined region will increase the chance of effective collisions between them [24]. Furthermore, rGO with better mobility of charge carriers can also become the outstanding media of efficient electrical communication between the enzyme and the electrode.

3.6. Selectivity of the nitrite biosensor

Selectivity is important in practical use of the biosensors. The selectivity of the biosensor is evaluated by using the amperometric current-time method among eight typical interfering substances (ascorbic acid (b), dopamine (c), uric acid (d), NaNO_3 (e), KCl (f), MgSO_4 (g), citric acid (i), acetic acid (j) solutions). As Fig. S9 shown, eight tested substances do not interfere the result of testing NaNO_2 significantly. This result can be attributed to the intrinsic selectivity of HRP for its substances.

3.7. Stability and reproducibility of the biosensor

Cycle stability is an important evaluation index for estimating the performance of biosensor. 100 continuous cyclic scans on $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ were repeated in the potential range from -0.7 to 0.1 V at a scan rate of 0.1 V s^{-1} (Fig. S10). No obvious change of CV curves is observed. Hence, the prepared $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ possesses excellent cycle stability.

The long-term stability of fabricated biosensors was studied by examining its current response after storage at 4°C . The biosensors could retain 94.1% of the initial response to NaNO_2 ($400 \mu\text{M}$) after four-week storage, demonstrating good long-term stability. The good long-term stability can be contributed to the good biocompatibility of Co_3O_4 nanosheets, which can provide a favorable microenvironment for HRP to retain its bioactivity.

The reproducibility of the biosensor was investigated by determining $100 \mu\text{M}$ NaNO_2 in PBS (pH 5.5). The relative standard deviation (R.S.D) is 3.2% for 5 successive measurements. Five biosensors prepared under the same conditions independently give a R.S.D. of 3.4% for the determination of $100 \mu\text{M}$ NaNO_2 in PBS (pH 5.5), indicating an acceptable electrode-to-electrode reproducibility.

3.8. Analysis of real samples

In order to evaluate the potential application of the sensor in real samples, the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$

was applied for determination of nitrite in environmental water samples without any other pretreatment (Fig. S11). Recovery tests and UV-vis spectrophotometry were used to examine the reliability and accuracy of this method. The results confirm that it is possible to determine the nitrite concentration in real samples by using the proposed method (Table 1)

4. Conclusions

In summary, porous Co_3O_4 hexagonal nanosheets with a well-defined morphology have been synthesized successfully via a hydrothermal process and followed by calcinations. Co_3O_4 nanosheets immobilizing HRP, together with rGO, have been used to modify glassy carbon electrode to fabricate a mediator-free biosensor for the detection of nitrite. Porous Co_3O_4 nanosheets with negative surface charges and biocompatibility are available for immobilizing HRP and retain HRP's enzymatic stability and activity. More importantly, the substrate can simply be entrapped by rGO with a high surface area, permitting the access of a large quantity of enzymes immobilized in the porous Co_3O_4 nanosheets. Concentration of substrate and enzyme in this confined region will increase the chance of effective collisions between them. Due to the above reasons, the mediator-free nitrite biosensors display an extremely low limit of detection (LOD) as $0.21\ \mu\text{M}$ and a limit of quantification as (LOQ) as $0.58\ \mu\text{M}$ and a wide linear range of 1–5400 M. This study indicates that $\text{Co}_3\text{O}_4/\text{rGO}$ composite film is a potential matrix for enzymes immobilization and the construction of direct electrochemical biosensor.

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Figure Caption

Scheme 1 Schematic illustration of the $\text{Co}_3\text{O}_4/\text{rGO}$ composite film immobilizing HRP.

Fig. 1 XRD of Co_3O_4 PCHNSs (a) and $\beta\text{-Co}(\text{OH})_2$ precursor (b).

Fig. 2 SEM and TEM images. Low- and high-magnification SEM images of $\beta\text{-Co}(\text{OH})_2$ precursor (A-B) and Co_3O_4 PCHNSs (D-E). Typical TEM micrographs of $\beta\text{-Co}(\text{OH})_2$ precursor (C) and Co_3O_4 PCHNSs (F). The inset is the HRTEM image of the sample.

Fig. 3 UV-vis spectra of $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ composite film (a) and HRP (b).

Fig. 4 Cyclic voltammograms of the $\text{Co}_3\text{O}_4/\text{rGO/GCE}$ (a), Nafion/HRP/GCE (b), $\text{Co}_3\text{O}_4\text{-HRP/GCE}$ (c) HRP/rGO/GCE (d), $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ (e), in 0.1 M pH 7.0 PBS with a scan rate of 0.1 V s^{-1} .

Fig. 5 Cyclic voltammograms of the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ in 0.1 M pH 7.0 PBS with increasing scan rates from 0.05 to 0.5 V s^{-1} . Inset: plot of cathodic and anodic peak current vs. scan rate.

Fig. 6 Cyclic voltammograms of the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ in 0.1 M PBS with different pH values from 6 to 8 (A). plot of cathodic and anodic peak current vs. pH values (B).

Fig. 7 (A) Cyclic voltammograms of the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ in 0.1 M pH 5.5 PBS containing 0 (a), $200 \mu\text{M}$ (b), $400 \mu\text{M}$ (c), $600 \mu\text{M}$ (d) and $800 \mu\text{M}$ (e) NaNO_2 with a scan rate of 0.1 V s^{-1} ; the inset: cyclic voltammograms of the $\text{Co}_3\text{O}_4/\text{rGO/GCE}$ in 0.1 M pH 5.5 PBS containing 0 (a) and $400 \mu\text{M}$ (b) NaNO_2 . (B) Variations of catalytic currents and peak potentials vs. pH of $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$.

Fig. 8 Typical current-time response of the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ at -0.70 V to successive addition of NaNO_2 in stirred 0.1 M pH 5.5 PBS. The inset: Plot of steady state current vs. NaNO_2 concentration.

Table 1. Results for determination of nitrite in environmental water

Scheme 1 Schematic illustration of the $\text{Co}_3\text{O}_4/\text{rGO}$ composite film immobilizing HRP

Fig. 1 XRD of Co_3O_4 PCHNSs (a) and $\beta\text{-Co(OH)}_2$ precursor (b).

Fig. 2 SEM and TEM images. Low- and high-magnification SEM images of β -Co(OH)₂ precursor (A-B) Co₃O₄ PCHNSs (D-E). Typical TEM micrographs of β -Co(OH)₂ precursor(c) and Co₃O₄ PCHNSs (F).The inset is the HRTEM image of the sample

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Fig. 5 Cyclic voltammograms of the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ in 0.1 M pH 7.0 PBS with increasing scan rates from 0.05 to 0.5 V s^{-1} . Inset: plot of cathodic and anodic peak current vs. scan rate.

Fig. 6 Cyclic voltammograms of the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ in 0.1 M pH 7.0 PBS with different pH

values from 6 to 8 (A). plot of cathodic and anodic peak current vs. pH values (B).

Fig. 7 (A) Cyclic voltammograms of the $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$ in 0.1 M pH 5.5 PBS containing 0 (a), 200 μM (b), 400 μM (c), 600 μM (d) and 800 μM (e) NaNO_2 with a scan rate of 0.1 V s^{-1} ; the inset: cyclic voltammograms of the $\text{Co}_3\text{O}_4/\text{rGO/GCE}$ in 0.1 M pH 5.5 PBS containing 0 (a) and 400 (b). (B) Variations of catalytic currents and peak potentials vs. pH of $\text{Co}_3\text{O}_4\text{-HRP/rGO/GCE}$.

Fig. 8 Typical current–time response of the Co₃O₄-HRP/rGO/GCE at -0.70 V to successive addition of NaNO₂ in stirred 0.1 M pH 5.5 PBS. The inset: Plot of steady state current vs. NaNO₂ concentration.

Table 1 Results for determination of nitrite in environmental water

Samples	UV-vis spectrophotometry (μM)	This work (μM)	Added (μM) ^a	Found (μM) ^b	RSD (%, n=5)	Recovery (%, n=5)
1	63.7	64.5	100	161.7	2.4	98.3
2	70.2	71.3	100	173.9	2.7	101.5
3	67.9	68.7	100	164.6	2.5	97.6

^a Standard nitrite solution added to the samples.

^b The total concentrations of nitrite after addition of standard nitrite solution to the samples.

Graphical Abstract