



Simple and sensitive nitric oxide biosensor based on the electrocatalysis of horseradish peroxidase on AuNPs@metal–organic framework composite-modified electrode

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Abstract

Fe-based metal–organic framework (MIL-101(Fe)) was synthesized through a simple solvothermal synthesis and then used to prepare the AuNPs-decorated MIL-101(Fe) nanocomposite (APPPM(Fe)) by a multi-step layer-by-layer assembly process. Benefited from the porous structure of MIL-101(Fe) and the multilayer assemble process, the loading amount of AuNPs on APPPM(Fe) was enhanced and exhibited a fine biocompatible interface and high conductivity. Through the intense Au–S bond, high loading amount of horseradish peroxidase was immobilized on APPPM(Fe) and the native bioactivity of HRP was kept to realize its direct electrochemistry. From the electrochemical kinetics, the constructed biosensor displayed fast electron transfer and good electrocatalysis activity for the detection of nitric oxide (NO) with wide linear range from 0.033 to 5370 μM and a low detection limit of 0.01 μM (3 σ) as well as fine stability, reproducibility and specificity. According to results of real sample analysis, the proposed electrochemical biosensor offers fast and simple detection of NO in real serum. Therefore, the present strategy definitely provided a potential application prospect in NO clinic detection and disease therapy.

Keywords Biosensor · Metal–organic framework · Nanocomposite · Horseradish peroxidase · Nitric oxide · Electrocatalysis

Introduction

Nitric oxide (NO) is one of the important signaling molecules in all vertebrates, which can adjust neural activity and blood flow [1]. A series of diseases and multisystem symptoms were caused or affected by NO production or signal transduction disorders which often exhibited an abnormal NO level [2, 3]. Hence, it was an effective way to understand the disease process status through monitoring the NO level of patient. On the other hand, some NO-based therapy

strategy had been gradually trying to apply in disease treatment based on the dynamic change characteristics of NO such as organic nitrate, sodium nitrate and inhaled nitric oxide (iNO) which had been used for the heart failure, pulmonary hypertension and COVID-19 [4–10]. In this situation, patient NO level monitor also was necessary for control the dosage of NO donor drugs because the exceeded NO will affect and even harm human body [11]. Therefore, it is necessary to develop a class of detection methods with high sensitivity, wide linear range and low detection limit for the detection of NO.

In the past decades, multiple analytical techniques have been developed for qualitative and quantitative analysis of NO or its derived oxides, for instance, high-performance liquid chromatography (HPLC) [12, 13], gas chromatography–mass spectrometry (GC–MS) [14, 15] and spectrophotometry [16–18]. However, these methods still presented several shortcomings, including complex sample pretreatment, time-consuming, high detection limit and complex operation. As a comparison, the electrochemical sensor is a supplementary analysis tool for NO detection because of the simplicity, rapidity, high sensitivity, high selectivity and real-time monitoring. Moreover, combined with the good

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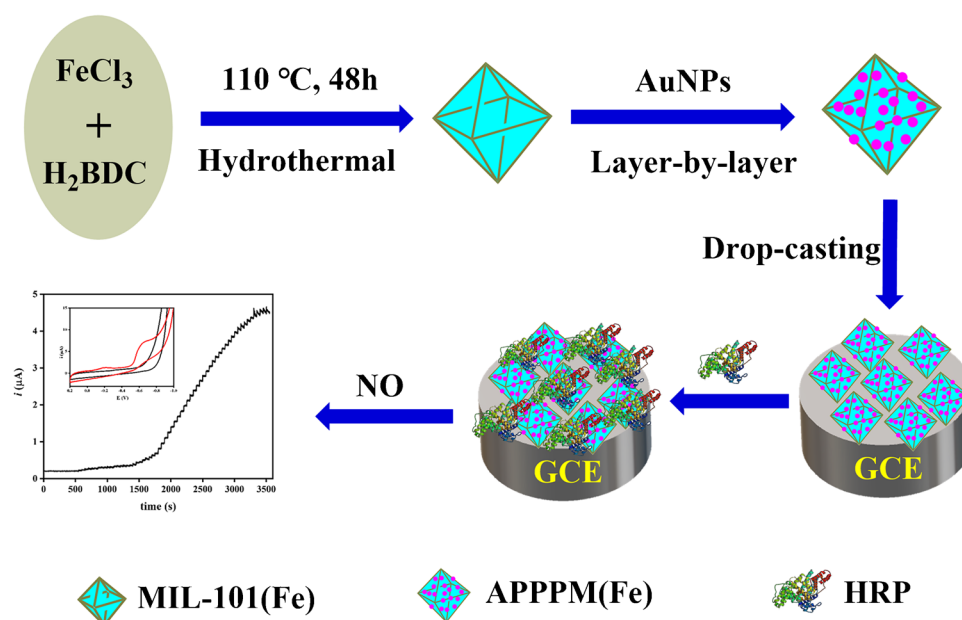
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Scheme 1 Schematic representations of the preparation of APPPM(Fe) and the construction of the nitric oxide biosensor



property of nanomaterial and fine specificity of biological enzyme, nanomaterial-based electrochemical biosensor had got more attentions and achieved more applications for the highly sensitive detection of target [19–22]. Metal–organic frameworks (MOFs) are a new class crystalline porous nanomaterial with regular three-dimensional framework. Due to their novel properties such as high surface area, ordered porosity, low density and tunable structures, they had got more attention in separation, gas storage, heterocatalysis, sensing and drug delivery [23, 24]. Benefited from the porous structure of MOFs, more active metal nanoparticles could be loaded on the surface of MOFs which could improve the conductivity of MOFs and immobilized the biological enzyme to keep its native activity; hence, MOFs may be a fine substrate of electrochemical biosensor for sensitive NO detection [25–29].

Herein, a simple and sensitive MOFs-based biosensing strategy was raised for the NO detection based on the electrocatalysis of horseradish peroxidase (HRP) (Scheme 1). In the strategy, Fe-based MOFs (MIL-101(Fe)) were synthesized through a simple solvothermal synthesis. After a multi-step layer-by-layer assembly process, AuNPs/PDDA/PSS/PEI/MIL-101(Fe) (APPPM(Fe)), a AuNPs-decorated MIL-101(Fe) nanocomposite, was achieved and then used for the immobilization of HRP through the intense Au–S bond. Benefited from the multilayer assemble process, the loading amount of AuNPs and HRP on MIL-101(Fe) was enhanced and can provide a fine biocompatible interface and high conductivity of APPPM(Fe) which could keep native bioactivity of HRP, and the constructed biosensor (HRP/APPPM(Fe)/GCE) displayed fast electron transfer and good electrocatalysis activity for the detection of NO with wide

linear range and low detection limit as well as fine stability, reproducibility and specificity. Therefore, this work opened a new way for the clinical applications of MOFs in electrochemical biosensors.

Materials and methods

Materials

Terephthalic acid (H_2BDC , 99.0%) was purchased from Shanghai Titan Scientific Co., Ltd (Shanghai, China). Polyethylenimine (PEI, branched, $\text{Mw} = 25,000$), poly (sodium 4-styrenesulfonate) solution (PSS, 30 wt% in H_2O , $\text{Mw} = 70,000$), poly (diallyldimethylammonium chloride) (PDDA, $\text{Mw} = 200,000\text{--}350,000$, 20 wt% in H_2O), $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, L-ascorbic acid, 3-hydroxytyramine and uric acid were purchased from Sigma-Aldrich (Shanghai, China). NaNO_2 , glucose, citric acid, L-cysteine, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, MgSO_4 and NaNO_3 were purchased from Chengdu Kelong Co., Ltd (Chengdu, China). Horseradish peroxidase (HRP) was provided by Sangon Biotech Co., Ltd (Shanghai, China). All reagents were used without further purification, and experimental water was ultra-pure water with a resistance of $18.2\text{ M}\Omega\text{ cm}$ by a Milli Q system. The preparation of AuNPs was based on a previous method listed in the literature [30].

Apparatus

Electrochemical measurements were performed on a CHI660 workstation (Shanghai Chenhua, China) with a conventional three-electrode system comprised of a platinum

wire employed as the counter electrode, a saturated calomel electrode (SCE) as the reference electrode and a modified glassy carbon electrode (GCE) as the working electrode. Scanning electron microscopy (SEM, Thermo scientific Apreo 2C, America), Fourier transform infrared spectrometer (FT-IR, Nicolet iS10, Thermo Fisher, America) and UV–visible spectrophotometer (Shimadzu, Suzhou, China) were performed to characterize the nanomaterials.

Preparation of APPPM(Fe)

The preparation of MIL-101(Fe) was based on the previous literature [31]. Next, 400 μL of PEI (20.0 mg mL^{-1}) aqueous solution was added to 2.0 mL of as-prepared MIL-101(Fe) (3.0 mg mL^{-1}). After shaking for 30 min, centrifugation with 10,000 rpm and three times of water washing were performed to separate the PEI functionalized MIL-101(Fe) (PEI/MIL-101(Fe)). Subsequently, 5 mg of PEI/MIL-101(Fe) was added into 3.0 mL PSS (w/v 0.5%) solution and shaken for 30 min. After centrifugation at 10,000 rpm for 10 min and washing with water, the obtained PSS/PEI/MIL-101(Fe) was added into another 3.0 mL PDDA (w/v 0.5%) solution and then shaken for 30 min. By the similar centrifugation and water washing process, the PDDA/PSS/PEI/MIL-101(Fe) was achieved. Next, the as-prepared electropositive PDDA/PSS/PEI/MIL-101(Fe) was transferred to 3.0 mL AuNPs solution followed with a 30-min shaking. After centrifugation and three times of water washing, the APPPM(Fe) composite was collected and re-dispersed in ultrapure water and stored in 4 °C for future use.

Construction of the biosensor

The GCE was first polished with 1.0, 0.3 and 0.05 μm alumina slurry successively, followed by ultrasonication thoroughly with ultrapure water and drying with nitrogen to get a mirror-like surface. Thereafter, 5.0 μL of the APPPM(Fe) (1.0 mg mL^{-1}) suspension was dropped onto the pre-treated GCE surface and dried naturally at room temperature. Finally, the electrode was immersed in 50 μL HRP (5.0 mg mL^{-1}) solution and stored at 4 °C for overnight to obtain the HRP/APPPM(Fe)/GCE-modified electrode. Before the as-prepared HRP/APPPM(Fe)/GCE biosensor was used for the detection, the modified electrode was rinsed with 1.0 mL of 0.10 M PBS (pH 7.4) for three times to remove loosely adsorbed HRP on the electrode surface.

Electrochemical measurements

All solutions for electrochemical analysis were N_2 -saturated, and we keep the N_2 atmosphere in whole measurement process. The direct electrochemistry of HRP/APPPM(Fe)/GCE was performed in 0.10 M PBS pH 7.4 by cyclic voltammetry

(CV) in the potential range from -0.8 to 0.2 V. Moreover, the electrocatalysis of HRP/APPPM(Fe)/GCE electrodes to NO that comes from the disproportionation reaction of NaNO_2 was performed in 0.10 M pH 2.0 NaAc-HAc buffer solution in the potential range from -1.0 to 0.2 V with the scan rate of 0.1 V s^{-1} . For amperometric measurement (i-t curve), the potential parameters were set as -0.630 V and the sensitivity of method was set as 10^{-6} . For real sample detection, 5.0 mL of 0.10 M pH 2.0 NaAc-HAc buffer solution was firstly added in a 10 mL electrolysis cell and purging by nitrogen under magnetic stirring. Meanwhile, the i-t curve electrochemical method was started to run to record the i-t curve until the i-t curve was steady to be a horizontal line and keeping 10 min. Then, 5 μL of the real sample was quickly added in the electrolysis cell. Finally, the current changing value in i-t curve after the adding of real sample was recorded to compute the NO concentration in real sample.

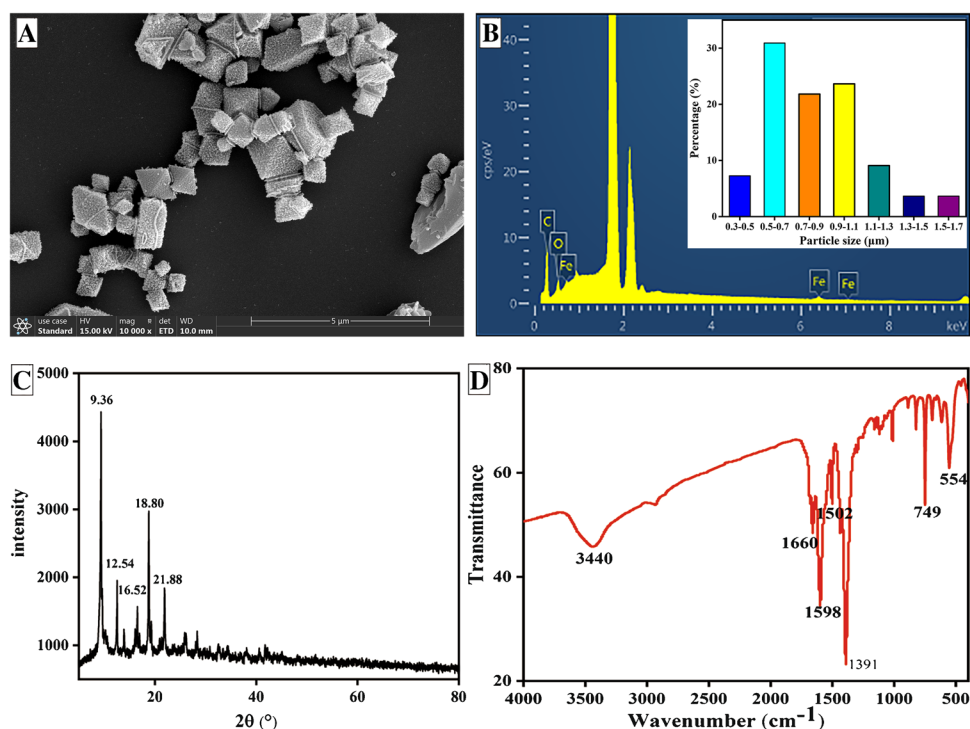
Result and discussion

Characterization

Different tools were used to characterize the morphology and composition of MIL-101(Fe). As can be seen from SEM image in Fig. 1A, MIL-101(Fe) presented a regular octahedron shape with relatively rough surface. From the EDS mapping of MIL-101(Fe) in Fig. 1B, the mean elements of MIL-101(Fe) were C, O and Fe with wt% of 63.19, 23.49 and 13.32, respectively. Moreover, according to the particle size distribution (measured from multiple SEM images) shown in the inset of Fig. 1B, the particle sizes of MIL-101(Fe) were mainly distributed in the range of 500–1100 nm. In addition, the XRD pattern of MIL-101(Fe) was also investigated as shown in Fig. 1C, and the main diffraction peaks were presented at 9.36°, 12.54°, 16.52°, 18.8° and 21.88° which revealed the successful formation of highly crystal MIL-101(Fe) and was corresponded to the previous study [32]. From the FT-IR of MIL-101(Fe) in Fig. 1D, the two strong absorption peaks at 1660 cm^{-1} and 1391 cm^{-1} indicated the existence of carboxyl group of H_2DBC . The characteristic peaks at 1598 cm^{-1} and 1502 cm^{-1} were corresponded to the C=C skeleton vibration of the benzene ring and 749 cm^{-1} was contributed to the bending vibration peak of the C-H. More importantly, the peak at 554 cm^{-1} could be assigned to the absorption of Fe–O which also proved the success preparation of MIL-101(Fe) [33].

To improve the conductivity of the nanomaterial and immobilize the HRP molecular to keep the native bioactivity, MIL-101(Fe) was modified with PEI, PSS and PDDA successively through a multi-step layer-by-layer assemble procedure and then decorated AuNPs on its surface to

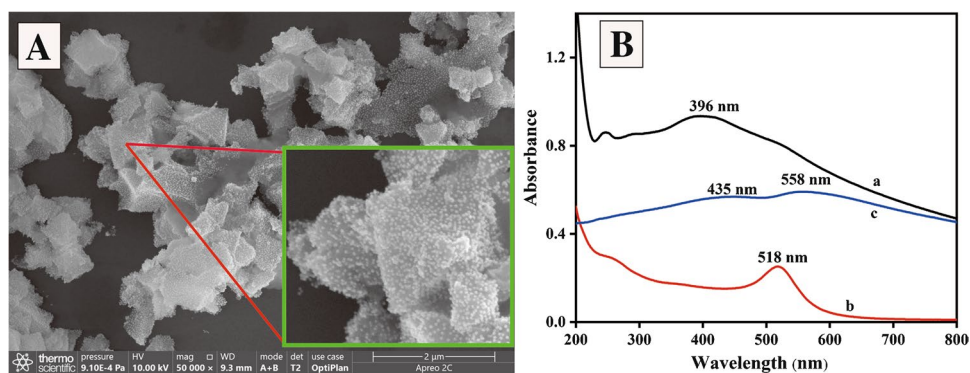
Fig. 1 **(A)** SEM image. **(B)** EDS mapping from SEM. Inset: the particle sizes distribution from SEM image. **(C)** XRD pattern and **(D)** FT-IR spectra of MIL-101(Fe)



obtain the APPPM(Fe) composite. The assemble process of APPPM(Fe) was characterized by zeta potential, and the result was listed in Fig. S1. It could be seen that the zeta potential of MIL-101(Fe) was 25.8 mV. After modified with PEI, the zeta potential of PEI/MIL-101(Fe) was increased to 37.7 mV due to the highly positive electricity of PEI. When negative PSS was warped on the surface of PEI/MIL-101(Fe), the zeta potential of PSS/PEI/MIL-101(Fe) changed to -35.8 mV. Then, followed with the modification of positive PDDA on the surface of PSS/PEI/MIL-101(Fe), the zeta potential of PDDA/PSS/PEI/MIL-101(Fe) reversed to 33.0 mV due to the intense positive electricity. Finally, after the assemble of negative AuNPs on the surface of PDDA/PSS/PEI/MIL-101(Fe), the zeta potential of the obtained APPPM(Fe) changed to -4.9 mV. Thus, it was convinced that the assemble process was successful and AuNPs had been successfully decorated on

PDDA/PSS/PEI/MIL-101(Fe) according to the above zeta potential characterization. From the SEM image of AuNPs@MIL-101(Fe) shown in Fig. 2A and inset, a large number of AuNPs were attached to the surface of MIL-101(Fe) without aggregation. Moreover, UV–Vis was also used to reflect the self-assemble process as shown in Fig. 2B. It could be seen that MIL-101(Fe) exhibited a strong and wide absorption band in the range of 220–500 nm. The absorption peaks at 247 nm and 396 nm with a slightly broad band were attributed to the characteristic absorption caused by ligand-to-metal charge transfer (LMCT) of O (II) $\rightarrow$ Fe (III), which confirmed the bonding of carboxylate oxygen to metal ion (curve a) [34–36]. And the characteristic absorption peak of AuNPs emerged at 518 nm caused by the surface plasma resonance (curve b). After the assemble of AuNPs on the MIL-101(Fe), the characteristic absorption peaks of MIL-101(Fe) and

Fig. 2 **(A)** SEM image of APPPM(Fe). Inset: the enlarged image. **(B)** UV–Vis spectra of (a) MIL-101(Fe), (b) AuNPs and (c) APPPM(Fe)



AuNPs were red-shifted to 435 and 558 nm, respectively (curve c), which was also the obvious evidence for the success of self-assemble process.

Direct electrochemistry of HRP on APPPM(Fe)

CV was employed to investigate the direct electrochemistry of HRP/APPPM(Fe)-modified electrode in 0.10 M pH 7.4 PBS buffer with a scan rate of 0.1 V s⁻¹. As shown in Fig. 3, no obvious redox peaks could be observed in bare GCE (curve a), MIL-101(Fe)/GCE (curve b) and APPPM(Fe)/GCE (curve c) in the potential window of -0.8–0.2 V. However, after HRP was immobilized on the surface of APPPM(Fe)/GCE electrode, a pair of stable, definite and reversible redox peaks at -0.415 V and -0.350 V were observed (curve d). Moreover, the potential difference (ΔE_p), an important parameter equal to the difference between the reduction peak and oxidation peak, was calculated to be about 65 mV which confirmed a fast electron transfer process between HRP and the APPPM(Fe)/GCE-modified electrode. This result indicated that the APPPM(Fe) supplied a bio-compatible interface for the immobilization of HRP to keep the native bioactivity of HRP.

In addition, the electrochemical kinetics was also investigated by scanning rate as shown in Fig. S2 which showed that the reaction process on the surface of this electrode belongs to the surface controlled quasi-reversible process. In accordance with Faraday's law, the surface concentration of electroactive HRP (Γ) at HRP/APPPM(Fe)/GCE

was calculated according to the following equation [37, 38]:

$$I_p = \frac{n^2 F^2 A \Gamma \nu}{4RT}$$

where I_p is the reduction peak current, n is the number of electrons, A is the surface area of the electrode, ν is the scan rate, and R , T and F are constants. The value of Γ was estimated to be 2.07×10^{-10} mol cm⁻², which was much greater than the theoretical monolayer coverage of the HRP (1.89×10^{-11} mol cm⁻²). This result hinted that HRP participated in the electron transfer process through multi-layer pattern on the AuNPs@MIL-101(Fe) composite film.

Electrocatalysis of HRP/APPPM(Fe)/GCE to reduction of H₂O₂ and NO

CV was applied to explore the electrocatalytic activity of HRP immobilized on the APPPM(Fe) nanocomposite for reduction of H₂O₂ and nitric oxide. As could be seen in Fig. S3, with addition of different concentrations of H₂O₂ in 0.10 M pH 7.4 PBS buffer (curve a to g), HRP/APPPM(Fe)/GCE exhibited an increased cathode current response which indicated that HRP/APPPM(Fe)/GCE had fine electrocatalytic activity for H₂O₂. Moreover, the electrocatalytic activity of HRP/APPPM(Fe)/GCE to NO was also performed by adding of varying concentrations of NaNO₂ in 0.10 M pH 2.0 NaAc-HAc buffer as presented in Fig. 4A. When without any addition of NaNO₂, only a pair of weak HRP redox peaks were observed at -0.150 and 0.210 V (curve a). However, when NaNO₂ was added in the acid buffer, NaNO₂ will experience a disproportionation reaction to release NO which could be catalyzed by the HRP on APPPM(Fe)/GCE and a well-defined cathode peak of NO at -0.630 V was emerged as well as the peak currents increased with the increase of NaNO₂ (curve b-d). Moreover, the electrocatalytic capacity of the APPPM(Fe)/GCE was also studied in 0.10 M pH 2.0 NaAc-HAc buffer containing NaNO₂ to compare with HRP/APPPM(Fe)/GCE. As shown in the inset of Fig. 4A, no any redox peaks were observed in the APPPM(Fe)/GCE which indicated that APPPM(Fe)/GCE had no electrocatalytic activity against NO (curve a). However, after the immobilization of HRP on APPPM(Fe), the obvious peak at -0.630 V proved the strong electrocatalytic ability of HRP/APPPM(Fe)/GCE to NO (curve b).

As shown in Fig. 4B, the amperometric current–time curve was utilized to investigate the steady-state response of HRP/APPPM(Fe)/GCE with successive injection of NaNO₂ in a N₂ saturated NaAc-HAc buffer (0.10 M, pH 2.0) at an applied potential of -0.630 V. The calibration curve shown in the inset of Fig. 4B illustrated a good linear relationship between the amperometric response and NaNO₂

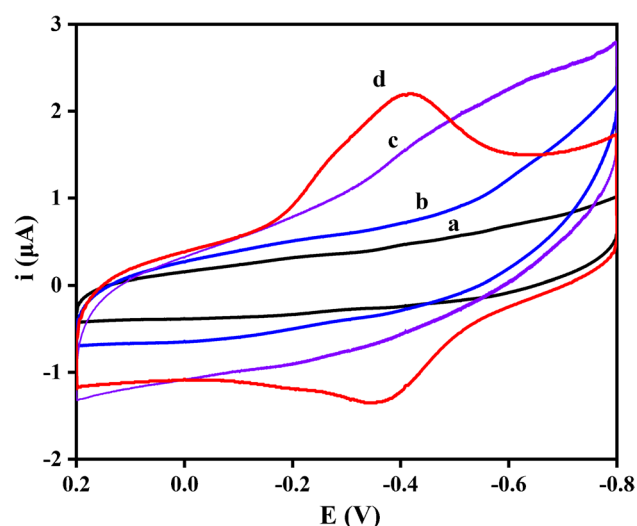


Fig. 3 CVs of (a) bare GCE, (b) MIL-101(Fe)/GCE, (c) AuNPs@MIL-101(Fe)/GCE and (d) HRP/APPPM(Fe)/GCE in 0.10 M pH 7.4 PBS at scan rate of 0.1 V s⁻¹

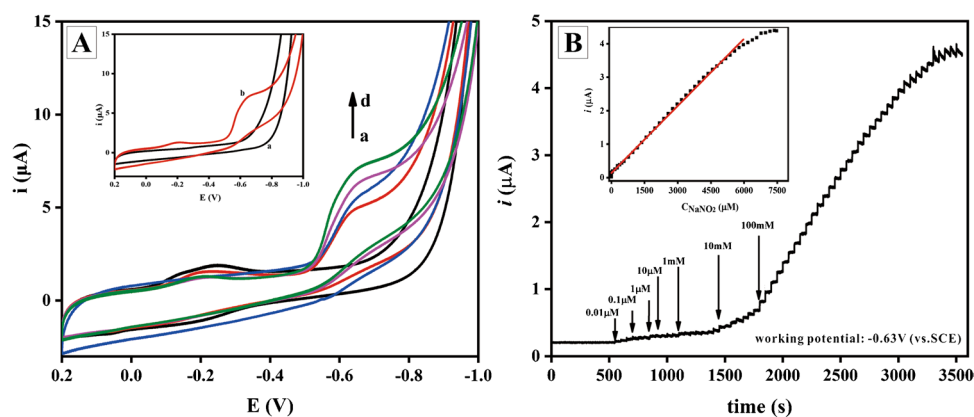


Fig. 4 (A) CVs of HRP/APPPM(Fe)/GCE in 0.10 M pH 2.0 NaAc-HAc buffer containing: (a) 0, (b) 100, (c) 200, (d) 300 and (e) 400 μM of NaNO_2 . Inset: CVs of (a) APPPM(Fe)/GCE and (b) HRP/APPPM(Fe)/GCE in 0.10 M pH 2.0 NaAc-HAc buffer containing 160 μM of NaNO_2 . (B) Typical amperometric current–time response

concentration in the range of 0.033–5370 μM with a lower limit of detection (LOD) of 0.01 μM at 3σ and the linear equation was $i = 6.686 \times 10^{-4} \times C_{\text{NaNO}_2} + 0.1380$ ($R = 0.9993$). In addition, as listed in Table S1 compared with numerous typical nitrite detection methods reported in literatures, the traditional methods of HPLC and UV–Vis exhibited narrow linear range and high detection limit. For the spectrophotometry, it could achieve lower detection limit but still narrow linear range than HPLC. However, for electrochemical method, both detection limit and linear range were improved and achieved very good results. Compared with other amperometry, the as-prepared biosensor in this experiment displayed a higher sensitivity and wider linear range, which can be attributed to the large specific surface area of MIL-101(Fe) and the high biocompatible surface of APPPM(Fe) to immobilize HRP and keep its native bioactivity.

Stability, reproducibility and specificity of HRP/APPPM(Fe)/GCE

Stability, reproducibility and specificity were the important aspects for the performance evaluation of the modified electrode. Fig. S4 displayed the consecutive 50 cycle CV scan curves of HRP/APPPM(Fe)/GCE in 0.10 M pH 2.0 NaAc-HAc buffer containing 160 μM NaNO_2 . Results showed that the percentage ratio of peak current decreased only 6.55% after 50 cycle which hinted the excellent stability of the biosensor. The reproducibility of the biosensor was estimated by analysis five HRP/APPPM(Fe)/GCE in 0.10 M pH 2.0 NaAc-HAc buffer containing 400 μM NaNO_2 . Results indicated that the relative standard deviation (RSD) of the five current responses was 2.46% which proved the biosensor possessed a favorable reproducibility for NaNO_2 detection. For specificity, a series of interference, including ascorbic acid (100 μM), uric acid

curve of the HRP/APPPM(Fe)/GCE biosensor upon successive addition of NaNO_2 into 0.10 M pH 2.0 NaAc-HAc buffer solution. Applied potential: -0.630 V. Inset: linear relationship between the amperometric response and NaNO_2 concentration

(100 μM), dopamine (100 μM), glucose (100 μM), citric acid (100 μM), L-cysteine (100 μM), MgSO_4 (100 μM), NaCl (100 μM) and NaNO_3 (100 μM), were selected to measure under same conditions and compared with NaNO_2 (10 μM). As shown in Fig. 5, with an excess (tenfold) amount of the inferences, the biosensor response exhibited superior selectivity for NaNO_2 which was contributed to the selective catalysis of HRP to NO produced from the disproportionation reaction of NaNO_2 under acid environment. This result demonstrate that the as-prepared biosensor has a good selective for NaNO_2 and the series of interference coexisted in serum had no or

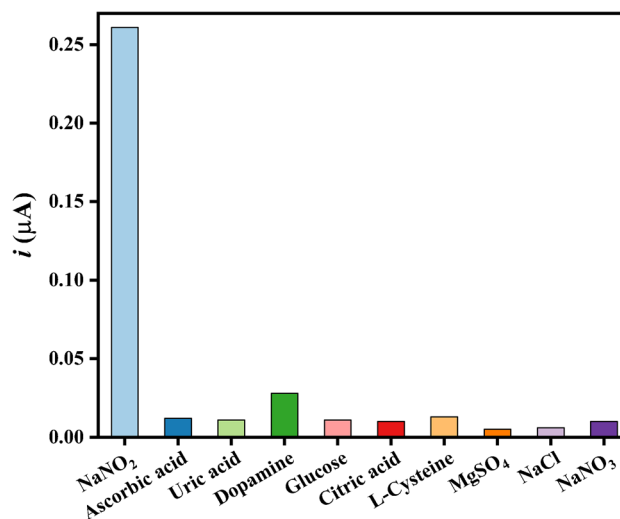


Fig. 5 Amperometric response of the proposed biosensor to NaNO_2 (10 μM), ascorbic acid (100 μM), uric acid (100 μM), dopamine (100 μM), glucose (100 μM), citric acid (100 μM), L-cysteine (100 μM), MgSO_4 (100 μM), NaCl (100 μM) and NaNO_3 (100 μM)

little influence to the detection of NaNO_2 in the pH 2.0 HAc-NaAc buffer under the working potential of -0.630 V.

Applications in sample analysis

The recovery experiment was carried out to evaluate the applied ability for real sample detection of the proposed biosensor. Three concentrations of 800, 1000 and 1200 μM of NaNO_2 standard solution were added into the undiluted serum sample (contained 82.3 μM of NO), respectively. Then 5 μL of above serum sample with standard addition was quickly added in the electrolysis cell which contained 5.0 mL of N_2 saturated 0.10 M pH 2.0 NaAc-HAc buffer solution and the i-t curve was measured using the proposed biosensor under the optimal condition. As listed in Table S2, the three standard addition serum samples were measured three times and averaged, respectively. The recovery ranged from 91.1% to 105.7%, and the RSD was between 5.00% and 7.33%. Therefore, the constructed biosensor could be a good selection for NO detection in serum.

Conclusions

In conclusion, AuNPs-decorated metal-organic frameworks nanomaterial was successfully prepared and applied for HRP immobilization and biosensor construction. Due to the high conductivity and fine biocompatible interface which was beneficial to keep the native bioactivity of HRP, the proposed biosensor displayed a good electrocatalysis activity for the detection of NO with wide linear range and low detection limit as well as fine stability, reproducibility and specificity. According to the real sample analysis, the proposed electrochemical biosensor can realize the fast, simple detection of NO in real serum. Therefore, the present strategy definitely provided a potential application prospect in NO clinic detection and disease therapy.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00604-022-05268-8>.

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Declarations

Conflict of interest The authors declare no competing interests.

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