NADH dehydrogenase-like behavior of nitrogen-doped graphene and its application in NAD\(^+\)-dependent dehydrogenase biosensing

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Abstract

A novel electrochemical biosensing platform for nicotinamide adenine dinucleotide (NAD\(^+\))-dependent dehydrogenase catalysis was designed using the nitrogen-doped graphene (NG), which had properties similar to NADH dehydrogenase (CoI). NG mimicked flavin mononucleotide (FMN) in CoI and efficiently catalyzed NADH oxidation. NG also acted as an electron transport “bridge” from NADH to the electrode due to its excellent conductivity. In comparison with a bare gold electrode, an 800 mV decrease in the overpotential for NADH oxidation and CoI-like behavior were observed at NG-modified electrode, which is the largest decrease in overpotential for NADH oxidation reported to date. The catalytic rate constant (\(k\)) for the CoI-like behavior of NG was estimated to be 2.3 \times 10^5 \text{ M}^{-1} \text{s}^{-1}, which is much higher than that of other previously reported FMN analogs. The Michaelis–Menten constant (\(K_m\)) of NG was 26 \mu M, which is comparable to the \(K_m\) of CoI (10 \mu M). Electrodes modified with NG and NG/gold nanoparticles/formate dehydrogenase (NG/AuNP/FDH) showed excellent analytical performance for the detection of NADH and formate. This electrode fabrication strategy could be used to create a universal biosensing platform for developing NAD\(^+\)-dependent dehydrogenase biosensors and biofuel cells.

1. Introduction

Nicotinamide adenine dinucleotide (NAD\(^+\))-dependent dehydrogenases have received considerable interest because of their promising electrocatalytic performance (Wang and Musameh, 2003; Wu et al., 2007; Zhang et al., 2007, 2004). Biosensors based on NAD\(^+\)-dependent dehydrogenases have been investigated for the detection of glucose (Deng et al., 2008; Zhang et al., 2007, 2004), ethanol (Guo et al., 2011; Wu et al., 2007) and lactate (Teymournian et al., 2012). These types of biosensors operate only when efficient recycling of the NAD\(^+\)/NADH cofactor system is achieved. Although the formal potential of the NAD\(^+\)/NADH couple is low (–0.56 V vs. SCE at pH 7.0, 25 °C), the oxidation of NADH is usually difficult to achieve at conventional electrodes and requires an overpotential as high as 1.0 V, which hampers the resulting biosensors’ practical applications (Jaegfeldt, 1980). Various methodologies have been developed to facilitate the electron transfer kinetics controlling cofactor recycling. For example, NADH dehydrogenase (CoI) is often utilized for recycling NAD\(^+\)/NADH in many biological redox systems (Li et al., 2008b; Sakai et al., 2009; Weiss et al., 1991). In CoI, the flavin mononucleotide (FMN) cofactor and an electron transport chain consisting of multiple iron–sulfur (FeS) clusters are of particular functional importance (Raffaelli et al., 2004): NADH binds to and delivers two electrons to FMN, which is consequently reduced to FMNH\(_2\). Then, the electrons are transferred via a series of FeS clusters from this redox group to the inner substrate ubiquinone (Weiss et al., 1991). Recently, an NADH sensor was constructed by utilizing phenazine derivative functionalized carbon nanotubes (CNTs) to mimic CoI for catalyzing NADH oxidation (Ma and Sim, 2012). In their study, the phenazine derivative acted as FMN, while CNTs acted as FeS clusters. Some FMN analogs, including flavins, quinones, phenoazine, phenothiazine and phenoxazine dyes, have been employed as redox mediators to reduce the overpotential of NADH oxidation (Itoh et al., 1998; Niculescu et al., 2003; Ramesh et al., 2003; Serban and El Murr, 2006). However, these mediators suffer from intrinsic disadvantages of limited stability and leaching from electrodes (Wooten and Gorski, 2010).
because NG exhibits much better electrocatalytic activity than graphene, since lone electron pairs of the nitrogen atoms can form a delocalized conjugated system with the \( sp^2 \)-hybridized carbon framework (Chen et al., 2012; Gong et al., 2009b; Wang et al., 2010; Zhang et al., 2008a). The nitrogen functionalities typically found in NG include pyridinic, pyrrolic and quaternary N (Scheme 1A) (Li et al., 2012, 2009; Wang et al., 2012; Wei et al., 2009). It has been reported that the pyridinic and quaternary N functionalities might play a particularly important role in governing the oxygen reduction reaction (ORR) (Geng et al., 2011). However, very few studies have reported the use of NG in catalyzing reactions involving biological molecules (Prathish et al., 2013). Because of the scarcity of studies in this field, the relationship between the nitrogen functionalities and the catalytic reaction mechanisms is still unclear.

In the present study, we found that NG had redox properties similar to CoI and acted as FMN, in which the pyridinic N (Scheme 1A) played a more important role, while a two-dimensional monolayer of carbon atoms packed into a dense hexagonal network structure acted as FeS clusters, so that the NG could efficiently catalyze NADH oxidation and serve as the electron transport "bridge" between NADH and the electrode (Scheme 1B). An 800 mV decrease in the over-potential for NADH oxidation and CoI-like behavior of NG were observed at NG-modified electrodes. By exploiting the CoI-like behavior of NG, we were able to recycle NAD\(^+\)/NADH and subsequently develop a novel electrochemical biosensing platform for NAD\(^+\)-dependent dehydrogenase catalysis. Formate dehydrogenase (FDH; Scheme 1C) was selected as a model NAD\(^+\)-dependent dehydrogenase, and a formate electrochemical biosensor was designed by a layer-by-layer (LBL) method. A hybrid layer consisting of a mixture of NG and gold nanoparticles (AuNPs) was coated onto a gold substrate and the electrode, and FDH was bound to the surface of the hybrid through a condensation reaction between terminal amino groups on the lysine residues of FDH and carboxyl groups on the AuNPs. The bound FDH and solution-phase NAD\(^+\) were used to catalyze the decomposition of formate, while NG recycled NAD\(^+\)/NADH. Compared to other reported dehydrogenase biosensors, the proposed formate biosensor exhibited good performance for the detection of formate. We concluded that this biosensor fabrication strategy could be used to create a universal biosensing platform for developing NAD\(^+\)-dependent dehydrogenase biosensors and biofuel cells.

2. Experimental

2.1. Materials and reagents

Formate dehydrogenase (FDH) from yeast (EC 1.2.1.2, and 15 U mg\(^{-1}\) solid), poly(diallyldimethylammonium chloride) (PDDA, 20%, w/w in water, MW = 200,000–350,000) were purchased from Sigma-Aldrich. Graphene oxide (GO) was synthesized from natural graphite flakes using a modified Hummers method (Hummers and Offeman, 1958). Graphene (G) was synthesized according to a previously published method (Li et al., 2008a). AuNPs were prepared according to the literature by adding sodium citrate solution to a boiling HAuCl\(_4\) solution (Zhang et al., 2008b). All other reagents were of analytical grade and were used without further purification. Ultrapure fresh water obtained from a Millipore water purification system (\( \geq 18 \) M\( \Omega \), Milli-Q, Millipore) was used throughout the whole experiment.

2.2. Apparatus

Field emission scanning electron microscopy (FESEM) images and high resolution transmission electron microscopy (HRTEM) images were measured by a HIFACHI S4800 SEM and a JEOL 2010 TEM, respectively. X-ray photoelectron spectroscopy (XPS) analysis was carried out on a Thermo Fisher X-ray photoelectron spectrometer system. Atomic force microscopy (AFM) images were performed in tapping mode using an Agilent 5500. Electrochemical measurements were performed on a CHI 660B workstation (Shanghai Chenhua Apparatus Corporation, China) with a conventional three-electrode system, which was composed of a platinum wire as the auxiliary electrode, a saturated calomel electrode (SCE) as the reference electrode, and an NG-, G or NG/AuNPs/FDH-modified gold electrode as the working electrode, respectively. A rotating disk electrode (RDE) system (Pine Research Instrumentation, USA) was used to evaluate the electrocatalytic rate constants of NG, and measurements were performed on a CHI 842B workstation. The RDE was a glassy carbon disk (5 mm diameter).

![Scheme 1](image-url)
NG was prepared according to a previously published procedure (Cao et al., 2013; Li et al., 2009) with some modifications. Details of the synthesis of NG and NG/AuNPs are displayed in Section 1 in Supporting information (ESI). The prepared NG samples were denoted as NG@500 °C@0.5 h, NG@700 °C@0.5 h, NG@900 °C@0.5 h, NG@1100 °C@0.5 h, NG@200 °C@4 h and NG@200 °C@8 h. Details of the preparation of NG-, G- and NG/AuNPs/FDH-modified electrode can be found in Section 1 in ESI.

2.4. Recovery experiment

The standard addition method was used to evaluate the accuracy and precision of measurements obtained with the formate biosensor. Samples (100 μL) of white vinegar and human serum, which were from the local market and from Nanjing University hospital, were diluted in 5 mL of phosphate buffer solution at pH 7.4. The diluted samples were spiked by appropriate amounts of formate, and recoveries were calculated.

3. Results and discussion

3.1. Characterization of NG

The FESEM and HRTEM images of NG in Fig. 1A and B show that the NG sheets were highly transparent and had wrinkles and folded regions, which indicated that the two-dimensional sheet structure of graphene was well maintained after N-doping. AFM was used to directly characterize the morphologies and layers of the NG sheets. Fig. 1C shows a representative AFM image of NG deposited on a mica substrate. Flattened NG sheets with an average thickness of about 1.0 nm were observed (Fig. 1D), which corresponds to fewer than three single graphene layers when considering the theoretical thickness of single-layer graphene (~0.34 nm).

XPS was used to further analyze the elemental composition and nitrogen bonding configuration in NG. The XPS spectra of pristine graphene and of NG over a wide range of binding energies (0–1000 eV) are shown in Fig. 1E. For pristine graphene, only those peaks that correspond to C 1s (284.5 eV) and O 1s (531.5 eV) signals were detected, whereas an additional N1s signal centered at 399.0 eV was observed for the NG samples (Gong et al., 2009a). The bonding configurations of N atoms in the NG samples were further investigated by high-resolution XPS (Fig. S1 in ESI). The high-resolution spectrum for N 1s could be fitted into three peaks at 398.5, 400.0 and 401.0 eV, corresponding to pyridinic, pyrrolic and quaternary N (Scheme 1A) (Klionsky et al., 2012; Li et al., 2009). Atomic percentages (at%) of N could be estimated from the peak areas of C 1s, O 1s and N 1s and their atomic sensitivity factors, while the frequency of pyridinic, pyrrolic and quaternary N was estimated from the corresponding relative areas obtained from curve fitting of the N 1s spectrum (Gong et al., 2009a). Table 1 displays the atomic content of N and its corresponding frequency of three different N configurations. It was found that the N content decreased with increasing annealing temperature, which may be because the self-released oxygen from graphene oxide burnt N at high temperature (Van Khai et al., 2012). The energy peak intensity of pyridinic N decreased with increasing annealing temperature, also suggesting that the pyridinic N structures could be decomposed at elevated temperature (Li et al., 2009).

3.2. Col-like behavior of NG

3.2.1. The oxidation of NADH at NG-modified electrodes

In order to verify the Col-like behavior of NG, the electrocatalytic activity of NG toward NADH oxidation was investigated. Cyclic voltammetry was first performed in the absence of NADH (Fig. 2A). The results reveal that one pair of redox peaks at ~−0.25 V appeared at the NG-modified electrode, while no obvious redox peaks were observed at the bare gold substrate electrode or graphene-modified electrode, which demonstrated the electrochemical activity of NG. The oxidation of NADH occurred at ~−0.05 V for the NG-modified electrode (Fig. 2B), in comparison with +0.75 V for the gold substrate electrode and +0.4 V for the graphene-modified electrode. Compared to the gold substrate electrode, the overpotential for NADH oxidation was
Table 1: The atomic contents of N, frequency of three different N functionalities in the NG samples and the corresponding NADH oxidation current (discussed in Section 3.2.5).

<table>
<thead>
<tr>
<th>Samples</th>
<th>N content [at%]</th>
<th>Frequency of pyridinic N (%)</th>
<th>Frequency of pyrrolic N (%)</th>
<th>Frequency of quaternary N (%)</th>
<th>ionic current [μA]</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG@1000°C 0.5 h</td>
<td>1.97</td>
<td>25.91</td>
<td>27.86</td>
<td>46.23</td>
<td>5.507</td>
</tr>
<tr>
<td>NG@900°C 0.5 h</td>
<td>2.98</td>
<td>27.93</td>
<td>33.89</td>
<td>38.18</td>
<td>9.421</td>
</tr>
<tr>
<td>NG@700°C 0.5 h</td>
<td>4.43</td>
<td>29.76</td>
<td>25.86</td>
<td>44.38</td>
<td>11.90</td>
</tr>
<tr>
<td>NG@500°C 0.5 h</td>
<td>5.11</td>
<td>28.77</td>
<td>29.19</td>
<td>42.04</td>
<td>13.01</td>
</tr>
<tr>
<td>NG@200°C 0.5 h</td>
<td>6.73</td>
<td>31.43</td>
<td>32.97</td>
<td>35.60</td>
<td>14.03</td>
</tr>
<tr>
<td>NG@200°C 1 h</td>
<td>8.17</td>
<td>36.03</td>
<td>25.13</td>
<td>38.84</td>
<td>14.27</td>
</tr>
</tbody>
</table>

* ionic current refers to the electrocatalytic current of NADH oxidation.

Fig. 2. Cyclic voltammograms recorded at the bare gold electrode and gold electrodes modified with NG or graphene in deoxygenated 0.1 M PBS (pH = 7.4) without (A) and with (B) 5.0 mM NADH. (C) CVs of different concentrations of NADH at the NG-modified electrode. Scan rate: 5.0 mV s⁻¹.

dramatically reduced by 800 mV at the NG-modified electrode, which is the largest decrease in overpotential for NADH oxidation reported to date. In addition, the initial potential for the onset of oxidation of NADH occurred at about −0.4 V, close to the formal potential of NADH/NAD⁺ (−0.56 V vs. SCE, pH = 7.0). Moreover, in the presence of higher concentrations of NADH (Fig. 2C), its oxidation peak current increased significantly and the reduction peak disappeared completely, indicating that NG could efficiently catalyze the oxidation of NADH in an ErCi-type catalytic reaction (Bard and Faulkner, 2001):

NG(red) → ne = NG(ox)  \hspace{1cm} (1)

NG(ox) + NADH → NG(red) + NAD⁺  \hspace{1cm} (2)

3.2.4. Apparent Michaelis–Menten constant (Kₘ) of NG

The apparent surface coverage (θ) of the prepared NG-modified electrode was estimated to be 4.51 ± 0.06 × 10⁻¹¹ mol cm⁻², using the equation θ = n²F²Aθcat/4RT (Bard and Faulkner, 2001). The electron transfer kinetics of NG were also evaluated in terms of the relationship between peak potential and scan rate (Section 3 and Fig. S2C in ESI). kₗ was calculated to be 5.03 s⁻¹, which revealed that the electron transfer kinetics of NG were very fast and similar to the redox behavior of FMN and its analogs (Bard and Faulkner, 2001).

3.2.5. Effect of nitrogen doping content and doping types on NG activity

To verify the NG activity depended on the nitrogen doping content in NG, the electrocatalytic activity of NG toward NADH oxidation was investigated. Fig. S5 displays the CVs of NG-modified electrodes for NADH oxidation. The results reveal that the NADH oxidation current increased gradually with increasing nitrogen content to 6.73% (Table 1 and Fig. S6A). However, the NADH oxidation current was almost unchanged when the nitrogen content was more than 6.73%. The nitrogen doping types in NG were mainly pyridinic N, pyrrolic N and quaternary N. To further understand which kind of nitrogen doping type played a significant role in NADH electrocatalytic oxidation, the relationship between the NADH oxidation current and the frequency of pyridinic N, pyrrolic N and quaternary N in NG is listed in Table 1. Table 1 reveals that the pyridinic N frequency increases with increasing nitrogen content in NG, that is, the NADH oxidation current depends on the frequency of pyridinic N to a certain extent besides the NG containing 4.43% N as an exception (Fig. S6B). No obvious dependence of NADH oxidation on the content of pyrrolic N and quaternary N species was observed. Therefore, we
believe that the pyridinic N combined with surrounding carbon atoms formed an FMN analog in NG (the blue circled area in Scheme 1A) and played an important role in the electrocatalytic oxidation of NADH. Meanwhile, similar to FeS clusters in CoI, a two-dimensional monolayer of carbon atoms packed into a dense hexagonal network structure played the role of the electron transport “bridge,” which transferred the electrons from the active site to the substrate electrode.

3.2.6. Catalytic rate constants (k) of NG

To evaluate the catalytic rate constant of NG toward NADH oxidation, we performed rotating disk electrode (RDE) measurements. The current–potential curves of NADH oxidation at different electrode rotation rates ($\omega$) are shown in Fig. 3A. As expected from theory, the limiting currents obtained on the NG-modified electrode increased with rotation speed. Fig. 3B shows a deviation of the linearity in the Levich plots obtained at three different concentrations of NADH (1 mM, 2 mM and 4 mM), which indicated the influence of the dynamic factors on electrode reaction. The Koutecky–Levich ($K$–$L$) equation was used to determine catalytic rate constants ($k$) (Oyama and Anson, 1980)

$$i_{\text{lim}}^{-1} = i_{\text{a}}^{-1} + i_{\text{rec}}^{-1} = \left( nF \alpha k r_{\text{cat}} C_{\text{NADH}}^{1/2} \right)^{-1} + \left( 0.62 nF A D_{\text{rec}}^{1/2} C_{\text{NADH}}^{1/2} \right)^{-1} \omega^{-1/2}$$

In accordance with the $K$–$L$ equation, at a given potential, a plot of $i_{\text{lim}}^{-1}$ versus $\omega^{-1/2}$ showed a linear relationship (Fig. 3C). At the intercept, $k$ was estimated to be $2.0 \times 10^5 \text{M}^{-1} \text{s}^{-1}$, which is much higher than previously reported values for FMN analogs, such as nile blue (1400 M$^{-1}$ s$^{-1}$), methylene green (11975 M$^{-1}$ s$^{-1}$), riboflavin (200 M$^{-1}$ s$^{-1}$), and meldola blue (19837 M$^{-1}$ s$^{-1}$), which have been applied for the electrocatalytic oxidation of NADH (Munteanu et al., 2001; Santos et al., 2002). These results suggest that NG could be a good electrocatalyst for NADH oxidation.

![Figure 3](image1)

**Fig. 3.** (A) Current–potential curves recorded at different electrode rotation rates for oxidation of 1 mM NADH. (B) Levich plots for the electrocatalytic response at 0.1 V vs. SCE for different concentrations of NADH. (C) Koutecky–Levich plots of NADH in pH=7.4 PBS at a scan rate of 5 mV s$^{-1}$.

![Figure 4](image2)

**Fig. 4.** (A) Chronoamperometric response of the NG sensing electrode in PBS (pH 7.4) on NADH at $-0.05$ V vs. SCE. (B) Plot of $\Delta I$ vs. NADH concentration. (C) Chronoamperometric response of the formate sensing electrode at a working potential of $-0.05$ V vs. SCE. Background electrolyte: 0.1 M PBS (pH=7.4) containing 2.0 mM NAD$^+$. (D) Plot of $\Delta I$ vs. formate concentration. Insets show the enlarged view of the defined segment. $\Delta I$ values are equivalent to the observed current minus the background current.
3.3. Application of NG with CoI-like behavior

3.3.1. Amperometric detection of NADH

Based on the excellent electrocatalysis of NG and reactions (1) and (2), the amperometric response of NADH on the NG-modified electrode was obtained at −0.05 V by spiking NADH aliquots into a stirred PBS buffer. The anodic current increased and reached a steady state within 5 s after the addition of NADH (Fig. 4A). The response consisted of three linear ranges (Fig. 4B). The first was from 1.00 to 100 μM with a linear equation of \( \Delta I = -0.0289c_{\text{NADH, μM}} + 0.124 \) (r = 0.9983); the second was from 0.100 to 4.00 mM with a linear equation of \( \Delta I = -6.28c_{\text{NADH, mM}} - 3.02 \) (r = 0.9955); and the third was from 4.00 to 30.0 mM with a linear equation of \( \Delta I = -0.779c_{\text{NADH, mM}} + 21.2 \) (r = 0.9981). The limit of detection (LOD) was 300 nM (S/N = 3). The selectivity and stability of the response of NADH on the NG-modified electrode were also examined. Glutathione (GSH), uric acid (UA) and dopamine (DA) each were added at 5 mM to the electrolyte solution, but did not interfere with the current response (Section 8 and Fig. S7 in ESI). A stable amperometric response was recorded over a continuous period of 2000 s (Fig. S7). Table S1 lists the response characteristics of the NG-modified electrode compared to other NADH sensors reported in the literature. Notably, our NG-modified electrode exhibited a wider linear range and lower overpotential for NADH oxidation compared to these other sensors.

3.3.2. Amperometric detection of formate

Since NG could efficiently catalyze NADH oxidation and FDH is a NAD⁺-dependent dehydrogenase, we used NG in place of Col to realize the recycling of NADH/NAD⁺ in an FDH catalytic system and developed a novel electrochemical biosensor for measurement of formate based on the NG/AuNPs/FDH electrode, which catalyzed reactions (1), (2) and (3) (Barman, 1969):

\[
\text{Formate} + \text{NAD}^+ \rightarrow \text{CO}_2 + \text{NADH} 
\]

In the NG/AuNPs/FDH electrode, AuNPs demonstrated that they almost did not affect the NADH oxidation (Section 10 and Fig. S8) and was used to bind FDH through a condensation reaction between terminal amino groups on the lysine residues of FDH and carboxyl groups on the AuNPs (Chen et al., 2011). For the prepared NG/AuNPs, FESEM and HRTEM showed that AuNPs were uniformly deposited onto the NG film and that the average size of the AuNPs was about 10 nm (Section 11 and Fig. S9A and S9C in ESI). After the NG/AuNPs-modified electrode was immersed in FDH solution for 24 h, the size of AuNPs became larger due to the bonding of FDH (Fig. S9B). X-ray powder diffraction (XRD) confirmed that the NG was effectively modified to yield an NG/AuNPs hybrid (Section 11 and Fig. S9D in ESI). Zeta-potential analysis was also used to verify the successful assembly of AuNPs on the NG (Section 11 and Fig. S9E in ESI). Electrochemical impedance spectroscopy (EIS) demonstrated the stepwise assembly of the NG/AuNPs/FDH electrode (Section 11 and Fig. S9F in ESI), and Fourier transform infrared spectroscopy (FT-IR) was used to further ascertain that FDH was bound to the AuNPs by means of a condensation reaction (Section 11 and Fig. S9G in ESI). The influences of the loading amount of the NG/AuNPs, pH, NAD⁺ concentration and temperature on formate response were investigated and optimal conditions were selected for the NG/AuNPs/FDH electrode (Section 12 and Fig. S10 in ESI). Fig. 4C displays the typical current-time (l–t) curve of the formate sensing electrode, which was obtained by successive addition of formate to 0.1 M PBS. Upon successive additions of formate, a steady-state current plateau was rapidly achieved, indicating that the NG/AuNPs/FDH electrode responded quickly to the target molecule. The relationship between the amperometric current and the concentration of formate exhibited two linear ranges (Fig. 4D). The first was from 1.00 to 100 μM with a regression equation of \( \Delta I = -0.0582c_{\text{formate, μM}} + 0.0384 \) (r = 0.9994), and the second was from 0.100 to 5.00 mM with a regression equation of \( \Delta I = -34.2c_{\text{formate, mM}} - 5.25 \) (r = 0.9962). The LOD of the formate biosensor was 300 nM (S/N = 3). When the concentration of formate was higher than 5 mM, the response curve tended to reach a plateau, showed characteristic of Michaelis–Menten kinetics. \( K_m \) was estimated to be 0.144 mM. We compared our formate sensor to others reported in the literature (Table S2), and we found that our sensing electrode showed a wider linear range, lower LOD and lower applied potential for formate detection. Although \( K_m \) values were not reported for the other formate biosensors listed in Table S2, our reported value of \( K_m \) was much smaller than other NAD⁺-dependent dehydrogenases, such as glucose dehydrogenase (9.9 mM) (Goran et al., 2013), thus suggesting that our formate sensing electrode exhibited a higher affinity for formate.

To characterize the stability and reproducibility of the NG/AuNPs/FDH-modified electrodes, six sensing electrodes were independently fabricated, and the current response of each was measured after long-term storage. The current response obtained after the sensing electrodes were stored at 4 °C for 4 months was 92.5% of the initial response, and the relative standard deviation (RSD) of the current response was only 3.12% (Fig. S11), which indicates that the NG/AuNPs might have provided a favorable microenvironment for FDH to retain its bioactivity. The selectivity of the NG/AuNPs/FDH-modified electrode was also investigated. The possible interferences (methanol, ethanol and acetic acid) at concentrations of 50 mM did not give an obvious amperometric current response at the working potential of −0.05 V vs. SCE (Fig. S12), indicating that our sensing system exhibited a high selectivity to formate over other possible interferences.

To estimate the applicability of our methodology as a broader biosensing platform, sample recovery by means of standard addition was performed. Formate concentrations that fell within the two linear ranges of the biosensor were added to these samples. Table S3 shows that the range of recovery was 98.2–104%, with RSDs of 3.33–4.05%, thus indicating that these sensors could be used for the detection of formate in real samples.

4. Conclusions

In summary, we observed Col-like behavior for NG, which made NG capable of efficiently catalyzing NADH oxidation. The overpotential for NADH oxidation at an NG-modified electrode was decreased by 800 mV in comparison with that on a bare gold electrode. The catalytic rate constant (k) for NG-catalyzed NADH oxidation was estimated to be \( 2.3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \), which is much higher than that of previously reported FMN analogs, and the Michaelis–Menten constant (\( K_m \)) was 26 μM, which is comparable to the \( K_m \) of Col (10 μM). Therefore, NG could serve as a mimetic Col for NADH oxidation. An NG/AuNPs/FDH hybrid electrode was developed and used as a formate electrochemical biosensor, which exhibited remarkable performance in the real-time detection of formate. This strategy could be utilized to create an effective biosensing platform for developing an NAD⁺-dependent dehydrogenase-based biosensors and biofuel cells.

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Appendix A. Supplementary information

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References