

Synergistic effect of pyrroloquinoline quinone and graphene nano-interface for facile fabrication of sensitive NADH biosensor

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ABSTRACT

A self-assembly composite of graphene-pyrroloquinoline quinone (PQQ) was fabricated and modified on glassy carbon electrode (GCE) for sensitive detection of nicotinamide adenine dinucleotide (NADH). Chitosan (CTS) was applied to disperse graphene to form a stable robust film on GCE. A synergistic effect between PQQ and graphene was observed during the electrocatalytic oxidation of NADH, with about 260 mV reduction in the oxidation potential and 2.5-fold increase in the oxidation current compared with those on the bare GCE. The electrochemical sensors based on the modified electrodes allowed the detection of NADH with a good linear dependence from 0.32 to 220 μM with a high sensitivity of 0.421 $\mu\text{A } \mu\text{M}^{-1} \text{ cm}^{-2}$ and a low detection limit of 0.16 μM ($S/N=3$). It could also eliminate the interference of electroactive substances like ascorbic acid (AA), uric acid, and dopamine and its derivatives. The outstanding performances of graphene-PQQ/CTS composite capable of improving the electrical conductivity and accelerating the electron transport suggested its promising applications for design of different graphene based composites used in electrochemical sensing and energy fields.

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1. Introduction

Nicotinamide adenine dinucleotide (NADH) is an important coenzyme in the electron transport chain inside cells (Kim et al., 2012). NADH/NAD⁺ as the substrate of dehydrogenase, approximately 300 species of dehydrogenase depend on the mutual transformation between them to carry out the catalytic function (Teymourian et al., 2012; Jaegfeldt et al., 1983). It involves in many metabolic processes, for example, it stimulates the generation of cellular neurotransmitters, provides energy for the immune system, and excites the function of brain and repairs the DNA system (Omar et al., 2016). Therefore, the efficient detection of NADH is crucial to the development of the extraordinary biosensors in view of the specific substrate based on dehydrogenase. Traditional detection methods, such as high-performance liquid chromatography (HPLC) (Ritov et al., 2004), colorimetry (Liang et al., 2015) and fluorometry etc. have been used to analyze NADH. Although these strategies have an impact on improving the detection sensitivity, there are still some drawbacks, i. e., high cost, complex preparation steps and tedious labors etc. Electrochemical sensors, because of their convenient operation, economical efficiency, rapid response, high selectivity and sensitivity (Chen and Shah, 2013; Sosna et al., 2013), are more likely to be a predominant method for

detection of NADH. Unfortunately, direct electron transfer of NADH on the traditional bare electrode is very slow, highly irreversible, which needs high activation energy, and produces high oxidation overpotential at 1.1 V on the carbon electrode (Moiroux and Elving, 1978) and 1.3 V on the platinum electrode (Jaegfeldt, 1980) at pH 7.0, respectively. More seriously, due to the generation of high oxidation potential, the oxidation products of NADH can be adsorbed on the surface of electrode, leading to the hindered reactions at electrode surface and electrode passivation (Jaegfeldt et al., 1983). All these issues result in the significant difficulty for real-time monitoring of NADH in biological samples (Kim et al., 2012).

Currently, it has been well known that use of mediators on the electrode could dramatically enhance the electron transfer rate of NADH, accompanied with the occurrence potential reduced. Since the overvoltage was related to the materials modified on the electrode, the surface modification designed to reduce NADH oxidation overpotential have received widespread attention (Sobczak et al., 2015; Rajaram et al., 2015). Various nanostructures, such as Co₃O₄ nanosheet (Chen et al., 2013), NiO nanoparticles (Sharifi et al., 2013), CdO nanoparticles (Pahlavan et al., 2014), ionic liquid/NiO nanocomposites (Karimi-Maleh et al., 2014) have also been applied to promote the process of NADH oxidation and to reduce the fouling of electrode.

Carbon based nanomaterials are widely used for preparation of NADH electrochemical sensors, including multilayered all-multi-walled carbon nanotubes (MWCNT) (Sun et al., 2013), Fe₃O₄

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nanoparticles/MWCNTs composite (Salimi et al., 2012), Poly(brilliant cresyl blue)/carbon nanotube composite (Yang and Liu, 2009), diphenylalanine peptide/MWCNT nanocomposite (Yuan et al., 2011), mesoporous carbon nitride (Zhang et al., 2014), electrochemically activated carbon materials (Li et al., 2014), DNA tetrahedron-graphene (Gr) composite (Li et al., 2015), Au-reduced Gr oxide nanocomposites (Govindhan et al., 2015), electroreduced Gr oxide-olothionine nanocomposite (Li et al., 2013a, 2013b), and Gr-Au nanorods nanosheets (Li et al., 2013a, 2013b). Among them, Gr is a novel carbon nanomaterial with monolayer of carbon atom arranged in sp^2 hybridized (Sangamithirai et al., 2015; Song et al., 2016), densely stack into a two dimensional honeycomb lattice (Yang et al., 2015; Liu et al., 2016), which has gained much attention since 2004 (Novoselov et al., 2004). Gr with high rate of electron transfer and low-resistance rate makes electron transfer easier and improves the electrical conductivity of the electronic component (Guo et al., 2011; Barsan et al., 2014; Liu et al., 2013). It has been reported to fabricate electrochemical transducers to extend sensitivity or amplify signal (Zhang et al., 2015; de Oliveira et al., 2015). Ayeshe et al. (2015) adopted Gr decorated with gold nanoclusters to selectively monitor trace Hg^{2+} in the water, approaching to 5×10^{-4} ppm. In addition, Gr has enormous specific surface area and smaller thickness, leading to its strong adsorption capacity (Tang et al., 2015). Many substances can be adsorbed by Gr, involving self-assembling to form hybrid materials. Jiang et al. demonstrated Gr could attach aryl groups and verified the strongest adsorption occurred at the edge of the Gr (Jiang et al., 2006).

Recently, researchers have focused on the employment of quinone, phenoxazine (Pinczewski et al., 2012) or phenothiazine as mediators to accelerate the oxidation of NADH. For example, electropolymerized 1,4-bis(3,4-dihydroxyphenyl)-2,3-dimethylbutane film exhibits excellent electrocatalytic performances towards the NADH oxidation (Ciszewski and Milczarek, 2000); the anodic current of NADH at polymerized film of toluidine blue O (TBO) increase remarkably in the presence of divalent cations (Ca^{2+} , Mg^{2+}), achieving on-line monitoring the electrocatalytic property of NADH oxidation (Zhang et al., 2010). Pyrroloquinoline quinone (PQQ), as a coenzyme of some bacterial dehydrogenases, mediates electron transfer and catalyzes oxidation of many substrates (Kumar and Kar, 2015; Guan et al., 2015). PQQ containing a segment of o-quinone, which owns a reversible and pH-dependent electron transfer between the oxidation and reduction form (Inoue and Kirchhoff, 2000), allowing the convenient electrochemical detection of NADH (Jao et al., 2007). Various methods have been applied to immobilize PQQ on electrodes. Immobilization of PQQ on polypyrrole (PPy) film/ glassy carbon electrode (GCE) maintains its

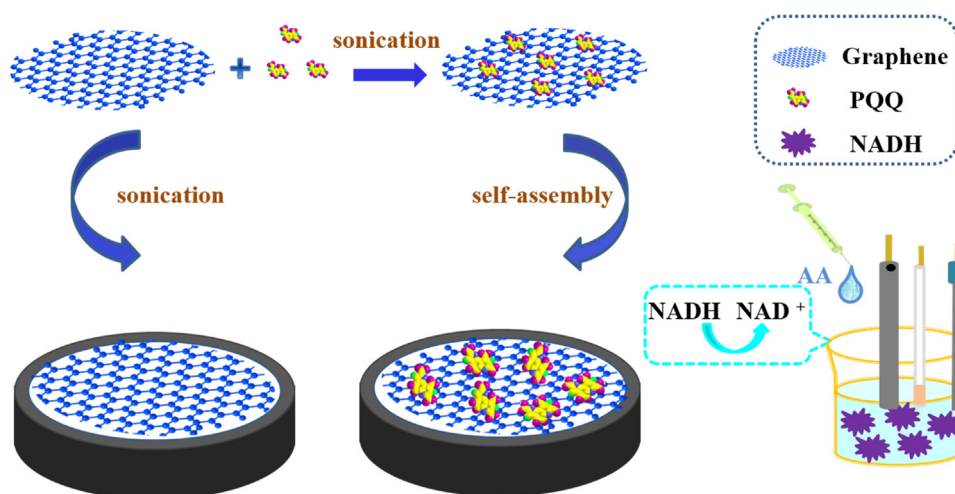
electron transfer processes still reversible (Inoue and Kirchhoff, 2000). It has been demonstrated that the appropriate thickness of PPy membrane can improve the selectivity and sensitivity of NADH oxidation. PQQ can also be immobilized on inorganic matrices such as silica gel decorated with zirconium oxide to realize the electro-catalytic oxidation of NADH (Yamashita et al., 2003). By carbodiimide coupling, PQQ could be covalently immobilized on cystamine assembled gold electrode (Katz et al., 1996). Although these strategies of immobilization of PQQ have made some achievements, the operation is complicated. The catalytic activity and stability of the modified electrodes still has certain gap compared with the practical application needs, despite the strategy to employ mediators to reduce the oxidation overpotential of NADH looks promising.

Hence, we demonstrate a very simple and facile method for immobilization of PQQ on GCE through adsorption. Firstly, the self-assembly composite Gr-PQQ/CTS was prepared and characterized. The electrocatalytic oxidation of NADH on the Gr-PQQ/CTS compound decorated GCE was presented. The prepared GCE modified with two-dimensional nano-interface of Gr-PQQ/CTS exhibits relatively lower potential of NADH oxidation and higher current response when compared with that of Gr/CTS decorated GCE. Moreover, the synergistic effect between Gr and PQQ in reducing the oxidation overpotential of NADH and their pH dependent electrochemical response are explored for the design of non-enzymatic NADH biosensor (Scheme 1).

2. Experimental

2.1. Principal materials

NADH and chitosan (CTS) was purchased from Sigma-Aldrich (St Louis, MO, USA). Gr (>98%), PQQ, ascorbic acid (AA), epinephrine bitartrate, norepinephrine bitartrate monohydrate, dopamine, levodopa, S-(-)-carbidopa, uric acid, 5-hydroxytryptamine (5-HT), and tyrosine was supplied with Aladdin Co. Ltd. (Shanghai, P. R. China). All other reagents used were of analytical level from Nanjing chemical reagent Co. Ltd. (Nanjing, P. R. China). Phosphate buffer solution (PBS, 0.2 M, pH 5.8 to 8.0) was prepared by mixing Na_2HPO_4 and NaH_2PO_4 solution and used as electrolyte. The NADH solution of 0.1 M, 40 mM and 20 mM were prepared and preserved at 4 °C. Deionized water (18.2 MΩ cm) obtained by Millipore-Q was used throughout unless specified.



Scheme 1. Schematic of GR-PQQ/CTS and GR/CTS modified GCEs Used for detection of NADH, investigating the Interference of AA to the NADH Oxidation.

2.2. Electrochemical detection and characterization

Electrochemical information was acquired by CHI660B electrochemical workstation (CHI Inc. USA) with three-electrode system, employing saturated calomel electrode (SCE) as a reference electrode, platinum filament as a counter electrode and GCE modified with G-PQQ/CTS or G/CTS as working electrode. The whole of amperometric experiments were carried out in 0.2 M PBS (pH 7.4) to stir. The electrochemical impedance spectrum (EIS) data were performed on Autolab PGSTAT302N (EcoChemie, Netherlands).

The ultraviolet-visible (UV-vis) spectrophotometer were carried out at room temperature on a Biomate 3S UV-vis spectrometer (Thermo, USA). The morphological characterization of G/CTS and G-PQQ/CTS were gained by scanning electron microscope (SEM, Zeiss, Germany).

2.3. Preparation of materials using in PQQ adsorption experiment

Gr of 1.0 mg/mL was fully ultrasonicated in deionized water. PQQ solution of various amounts were mixed with Gr solution and the final Gr concentration was 0.5 mg/mL. These materials were all followed by 90 min ultrasonic bath. Next, the gained solutions were centrifuged at speed of 8500 rpm for 20 min and then pipetted the supernatant to detect their ultraviolet absorption, respectively.

2.4. Preparation of Gr-PQQ/CTS self-assembly hybrid materials

CTS solution of 1% was prepared by ultrasonic dispersion in 60 μ L dilute acetic acid (1%, v/v), and PQQ solution of various volumes were added to 2 mL Gr solution of 0.5 mg/mL, respectively. The hybrid Gr-CTS and Gr-PQQ materials both followed by 90 min ultrasonic bath. Next, the gained various Gr-PQQ solution were centrifuged at exact same parameters as before and then washed with deionized water for 3 times. Finally, the materials obtained above were re-dispersed in deionized water and then added 30 μ L 1% CTS, following with 90 min ultrasonic bath to obtain the self-assembly composite materials.

2.5. Fabrication of Gr-PQQ/GCE film-modified GCE

The GCE (diameter 3 mm) was polished with 0.05 μ m alumina slurry and then thoroughly rinsed with water before surface

modification. The Gr-PQQ/GCE was fabricated by the self-assembly method between G and PQQ. The composite materials of 5 μ L containing various contents of PQQ were cast on GCE and dried in the air at room temperature. On the other hand, as the controlled groups, the electrodes modified with only Gr-CTS or PQQ were prepared on the paralleled processes by casting with equal quantity of solutions needed. Finally, the resulting modified electrodes were rinsed with PBS to wipe off unadsorbed materials.

2.6. Biological sample analysis in human serum

Human serum samples were obtained from a local hospital, and refrigerated at -20°C before use. The serum samples were then diluted by 100 times with 0.2 M PBS (pH 7.4). The stock solution for recovery tests of NADH was prepared by mixing 200 μ L diluted serum samples and 200 μ L NADH of 40 mM. The tests were performed by current-time method and the recovery was evaluated by: $\text{Recovery\%} = (\text{titration slope of NADH in stock solution}) / (\text{titration slope of NADH in PBS}) \times 100\%$.

3. Results and discussion

3.1. PQQ adsorption on Gr

The UV-vis absorption spectrum clearly proved the adsorption of PQQ on Gr. It could be seen from Fig. 1A that PQQ had two typical absorption peaks at 249 nm and 325 nm, with a shoulder peak at 275 nm. All these absorption peaks disappeared after treatment with 1 mL Gr solution of 0.5 mg mL $^{-1}$. The maximum adsorption amount of PQQ appeared at ~ 50 μ L, corresponding to 25 μ g PQQ totally adsorbed on 0.5 mg Gr (Fig. 1B), i.e., a loading efficiency of $\sim 4.8\%$. The further addition of PQQ hardly changed the adsorption amount. These results demonstrated that PQQ can be rapidly and efficiently assembled on Gr.

3.2. Characterization of Gr-PQQ/CTS on GCE

The SEM images displayed in Fig. S1 revealed the morphology of materials modified on the electrode. Fig. S1 A and B showed the Gr-PQQ/CTS and Gr/CTS modified on the GCE, respectively. Wrinkled and crumpled nanostructures were observed on both surface and these modified GCE could not be distinguished from the morphological status. The EDS results (Fig. S2) showed that the

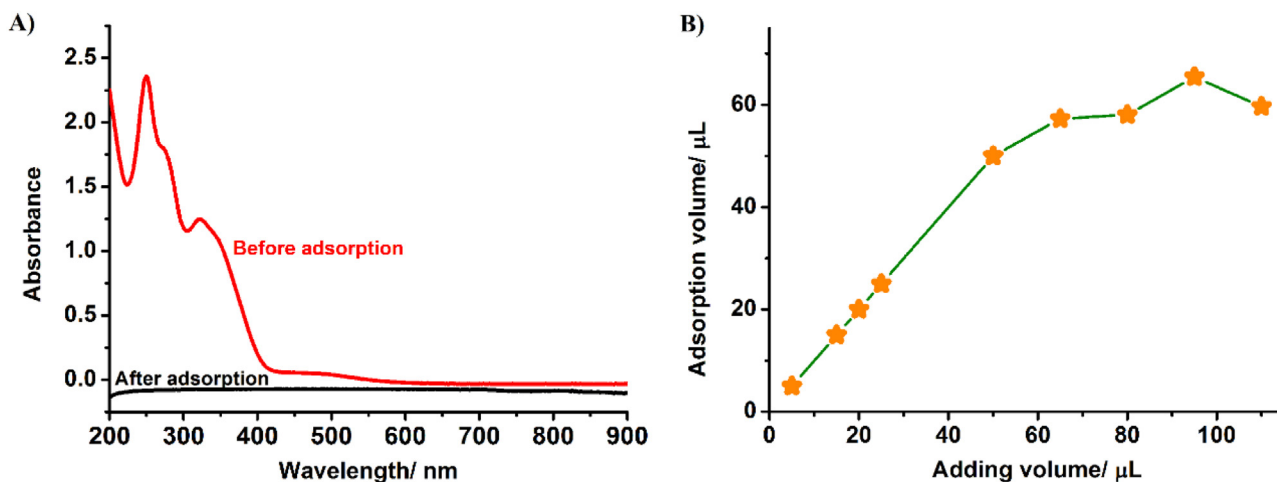


Fig. 1. (A) The UV-vis absorption spectra of 25 μ g/mL PQQ aqueous solution (red line) and the supernatant after treatment of 25 μ g/mL PQQ by 0.5 mg Gr (black line). (B) The adsorption curve of adding PQQ volumes in the corresponding Gr solution versus calculated relevant adsorption quantity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

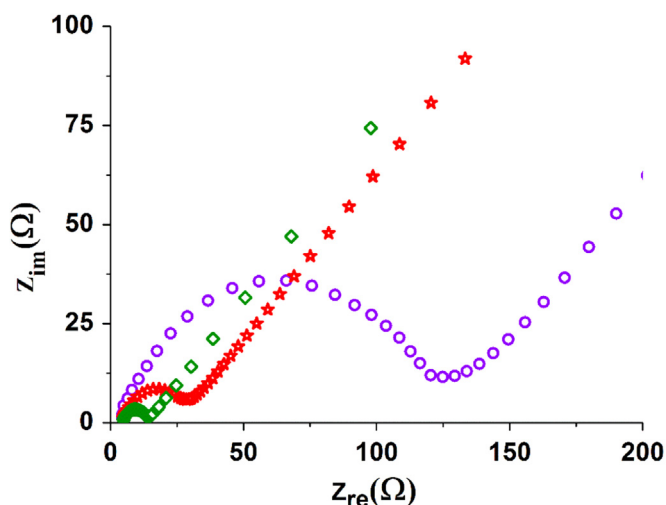


Fig. 2. Nyquist plots on bare GCE (violet, hollow round), Gr/CTS (olive, hollow square) and Gr-PQQ/CTS (red, solid star) -modified GCE. Electrolyte: 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ containing 0.1 M KCl. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nitrogen content is $\sim 5.0\%$ for the composite Gr-PQQ/CTS material and $\sim 3.5\%$ for Gr/CTS. The nitrogen content is differential dramatically between the two hybrid materials. It is implied that PQQ can be absorbed by Gr.

It has been reported that chitosan is a natural polymer with the ability of forming a stable water-insoluble uniform film on the electrode (Kang et al., 2009). In addition, homogeneous dispersion of Gr in Gr-PQQ/CTS could improve the mechanical strength of the modified film (Stankovich et al., 2006), leading to the formation of a robust Gr-PQQ/CTS film. Afterwards, we measured the electrochemical properties of Gr-PQQ/CTS by means of electrochemical impedance spectrum.

Electrical impedance was an appropriate mean for characterization of the heterogeneous electron transfer on the surface of the electrode. The information about the impedance of the electrode and the resistance of charge transfer (R_{ct}) on the electrode surface through the EIS were obtained. Fig. 2 displayed the typical Nyquist plots for the Gr-PQQ/CTS, Gr-PQQ/CTS film-decorated GCE and bare GCE in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ containing 0.1 M KCl. It has been observed that the R_{ct} value is about 120 Ω for the bare GCE (violet, hollow round). However, smaller semicircles had been seen in the Gr-PQQ/CTS (red, solid star) and Gr-PQQ/CTS (olive, hollow square) film-decorated GCE with an R_{ct} of 27 Ω and 9 Ω , respectively. It demonstrated that the redox reaction of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ was conducted easier on Gr-PQQ/CTS modified surface, indicating Gr-PQQ/CTS modified film could accelerate the surface charge transfer and diminish the energy barriers of the heterogeneous reaction on the electrode. Here, a faster electron transport rate and upper electrochemical property were obtained with Gr-PQQ/CTS modified GCE.

3.3. Electro-catalytic oxidation of NADH at Gr-PQQ/CTS film modified GCE

The bare electrodes such as carbon or platinum electrodes come up with poisoning effect in the process of electrocatalytic oxidation of NADH, leading to the high overpotential of NADH oxidation and undesirable fouling on electrodes (Moiroux et al., 1978; Jaegfeldt, 1980; Govindhan et al., 2015). Here, the function of Gr/CTS on the electrochemical oxidation processes was firstly investigated in PBS containing 1 mM NADH (Fig. 3A). The potential of NADH at 1 mM was 0.58 V on bare GCE in PBS. In contrast with the bare GCE, the oxidation peak current increased by 91.22% on

the Gr/CTS modified electrode according to cyclic voltammetry (CV) method at a scanning rate 100 mV s^{-1} , indicating the enhanced catalytic oxidation of NADH on Gr/CTS modified GCE surface. Also, the potential of NADH oxidation was 0.37 V on this modified GCE, corresponding to a negative shift by 210 mV compared to bare GCE. Since no such potential shift effect was observed for chitosan modified GCE, the presence of Gr made the oxidation overpotential of NADH reduced greatly. Thus the uniform Gr/CTS membrane on the electrode surface increased the oxidation peak current of NADH by promoting the electron transfer rate and accelerate the NADH catalytic oxidation.

Next, we tended to observe the effect of the modified Gr-PQQ/CTS film of electrocatalytic oxidation of NADH on GCE. The anodic peak current increased to ~ 2.5 fold at Gr-PQQ/CTS film-modified GCE comparing to the bare GCE, suggesting the even faster electron transfer rate and more excellent catalytic ability towards NADH oxidation. Moreover, the oxidation potential of NADH significantly reduced to 0.32 V, with a negative shift of about 260 mV after modified Gr-PQQ/CTS film. (Fig. 3A). This value was even 50 mV lower than that on the Gr/CTS modified electrode. Considering that potential of NADH was ~ 0.58 V on PQQ modified GCE, it could be deduced that the overpotential reduction about electrocatalytic oxidation of NADH should be attributed to the synergistic effect of Gr and PQQ.

To measure the effective electrode surface area, the cyclic voltammogram of the Gr-PQQ/CTS film or Gr/CTS modified GCE and bare GCE in 1 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ solution containing 0.1 M KCl (Fig. 3B). The formal potentials of $[\text{Fe}(\text{CN})_6]^{3-}$ redox peaks were roughly similar among these modified electrodes. The specific surface area of the electrodes could be acquired by Randles-Sevcik equation (Velasco, 1997):

$$I_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} v^{1/2} C$$

where, I_p represents peak value of current (A); A represents the specific surface area of the electrode (cm^2); D represents diffusion coefficient of the probe (the value for $\text{K}_3[\text{Fe}(\text{CN})_6]$ is $6.7 \times 10^{-6} \text{ cm}^2/\text{s}$ under 25°C); n represents the transferred electron number when occurs the redox reaction; v represents the scanning rate (V/s) and C represents the probe concentration (mol/cm^3). The measured effective surface areas were calculated to be 0.067 cm^2 , 0.112 cm^2 , and 0.179 cm^2 for bare GCE, Gr/CTS film modified GCE, and Gr-PQQ/CTS film modified GCE, respectively, demonstrating that the modification of Gr could make the active surface area of GCE greatly increased and the surface area could further expand after adsorption of PQQ.

CV curves with different scanning rates were explored in order to determine that the NADH oxidation was controlled by diffusion or adsorption processes on the Gr-PQQ/CTS film modified electrode (Fig. 3C). The current of NADH catalytic oxidation increased with the increasing scanning rate. A good linear relationship of peak current versus scan rate square root was obtained from 10 mV/s to 100 mV/s (Fig. 3D). It was inferred that the diffusion processes of NADH controlled the reaction on the electrode. According to this relationship, electron transfer process of NADH electrocatalytic oxidation (Fig. S3) on the Gr-PQQ/CTS film modified GCE, Gr/CTS film modified GCE and bare GCE could be quantitatively calculated by homogeneous reaction (k_s) as followed equation (Maiyalagan et al., 2013):

$$k_s = 1.11 D_0^{1/2} (E_p - E_{p/2})^{-1/2} v^{1/2}$$

where k_s means standard heterogeneous rate constant (cm/s); D_0 means apparent diffusion coefficient (cm^2/s); E_p means the oxidation peak potential (V); $E_{p/2}$ means the potential of half-wave oxidation peak (V) and v means the scan rate (V/s). In order to determine the value of k_s , we needed to obtain the diffusion

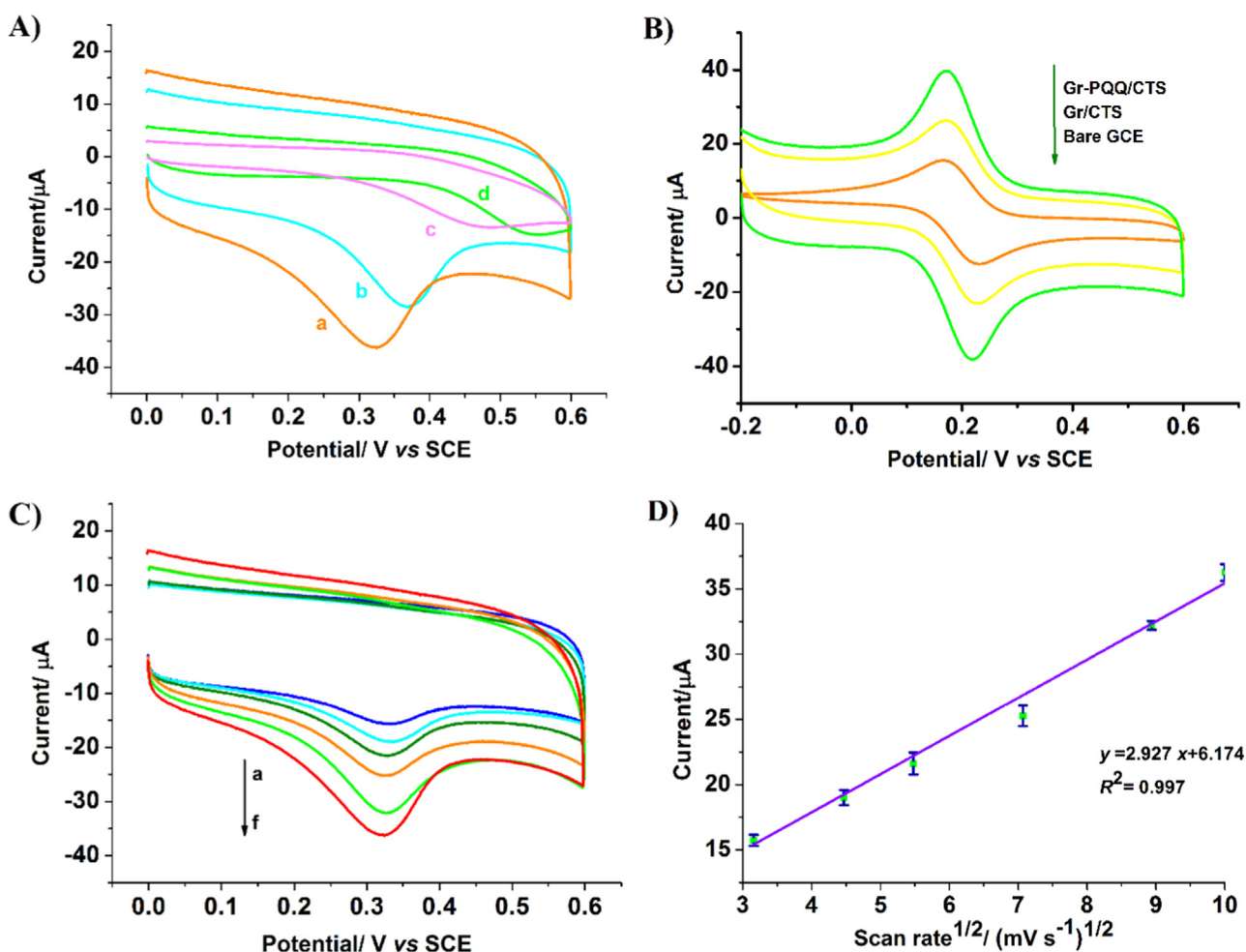


Fig. 3. (A) CV study of 1 mM NADH (0.2 M PBS, pH 7.4) at the Gr-PQQ/CTS (a), Gr/CTS (b), PQQ (c) modified GCE and bare GCE (d). The scan rate is 100 mV s⁻¹. (B) CVs of bare, Gr/CTS and Gr-PQQ/CTS modified GCE in 1 mM [Fe(CN)₆]³⁻ containing 0.1 M KCl. (C) CVs of Gr-PQQ/CTS modified GCE of 1 mM NADH (0.2 M PBS, pH 7.4) at the scan rate of 10 (a), 20 (b), 30 (c), 50 (d), 80 (e) and 100 (f) mV/s. (D) Linear relationship between the peak currents and the square root of scan rates.

coefficient of NADH earlier. The diffusion coefficient was gained based on the chronoamperometric results, according to the slope of current versus time^{-1/2} by the Cottrell equation. The calculated value of D_0 was 7.61×10^{-5} cm²/s for NADH. Ultimately, the k_s values of the NADH catalytic oxidation on the Gr-PQQ/CTS film modified GCE, Gr/CTS film modified GCE and bare GCE were measured to be 5.8×10^{-4} cm/s, 5.4×10^{-5} cm/s, 2.7×10^{-6} cm/s, respectively, indicating that the faster process of NADH catalytic oxidation on the Gr-PQQ/CTS film modified GCE than Gr/CTS modified GCE and bare GCE. All these results demonstrated that the modified film Gr-PQQ/CTS could provide a relatively fast electron transfer path, making the process of the NADH catalytic oxidation be accelerated greatly.

3.4. Optimization experiment of Gr-PQQ/CTS composites modified electrodes

Different PQQ amount of composite materials and pH of PBS had a significant influence on NADH detection. Fig. 4A displayed that different NADH oxidation peak currents appear on the modified composite materials containing various mass ratio of PQQ/CTS. The peak current reached maximum when mass ratio of PQQ/CTS was 1:100. The oxidation peak current decreased when continued to increase the PQQ mass ratio. The same results were achieved through amperometric method (Fig. S4). Therefore, the material with the best ratio of 1:100 was used in my next experiment.

The influence of PBS pH to the electrochemical signals of NADH was shown in Fig. 4B. The NADH oxidation peak current varied from pH 5.8–8.0, achieving a maximum value at pH 7.4 (Fig. 4C). It was also noteworthy that the employed pH of PBS also had an impact on the potential of NADH oxidation (Fig. 4C). Coincidentally, the optimized peak potential (0.32 V) were obtained at pH 7.4. To summarize, the composite material with the PQQ/Gr mass ratio of 1:100 and PBS of pH 7.4 were chosen in the following study.

3.5. The anti-interference ability, reproducibility and stability of Gr-PQQ/CTS modified GCE

The interference of bioactive substances such as AA could not be ignored, especially applying to the electrochemical analysis in biological samples. The response of CV was performed on Gr-PQQ/CTS modified GCE in the mixed solution composed of 0.5 mM AA and different levels of NADH. For the Gr-PQQ/CTS modified GCE, the obvious gap of the two oxidation peak was represented with the oxidation potential of 0 V and 0.32 V, relative to the oxidation of AA and NADH, respectively (Fig. 5A). Fig. 5B showed that the oxidation peak current was linearly dependent on the concentration of NADH in the presence of AA ($R^2 = 0.964$). In addition, it was worth noting that NADH oxidation peak current value was -36.4μ A and -36.8μ A corresponding to 1 mM NADH and in the mixture containing 1 mM NADH and 0.5 mM AA, revealing the

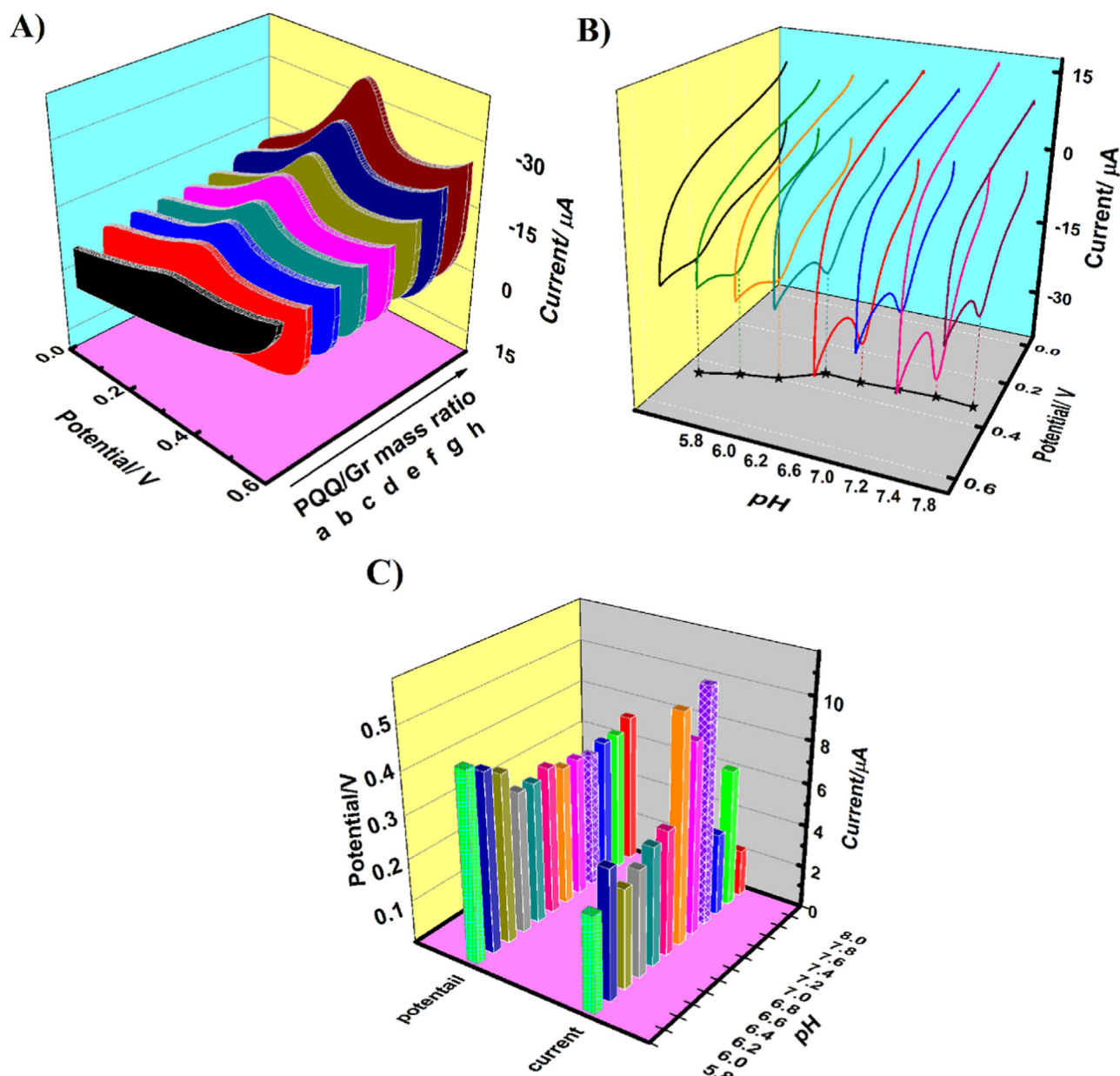


Fig. 4. (A) CV response of 1 mM NADH on Gr-PQQ/CTS composites with PQQ/Gr mass ratio of 1:1000 (a), 1:500 (b), 3:1000 (c), 1:250 (d), 1:200 (e), 1:40 (f), 3:200 (g), 1:100 (h) in PBS (pH 7.4). (B) PBS pH dependence of 1 mM NADH on Gr-PQQ/CTS film-modified GCE. (C) The comparison of NADH oxidation potentials and peak currents with pH from 5.8 to 8.0.

negligible influence in the presence of AA. Besides AA, the possible interference by electroactive neurotransmitters and metabolites, namely, epinephrine, norepinephrine, dopamine, levodopa, carbidopa, uric acid, 5-HT and tyrosine were evaluated (Fig. S5–S12). No significant interference was observed due to the differential peak potentials between NADH and interferents. This demonstrates that the co-existence of common small biomolecules hardly impacts on electrochemical analysis of NADH.

The reproducibility of Gr-PQQ/CTS GCE were measured through preparing of five Gr-PQQ/CTS composites electrodes. The tests of reproducibility were performed on the equal experimental conditions. The relative standard deviation (RSD) was discovered to $\sim 4.59\%$ based on the independent Gr-PQQ/CTS film-modified GCEs indicating an excellent reproducibility for the sensor of the NADH detection. The stability of the fabricated NADH sensor was measured via the current response to 1 mM NADH using the amperometric method. Based on the variation of the current

response, the sensitivity at Gr-PQQ/CTS modified GCE decreased to $\sim 89\%$ comparison of its original sensitivity and maintained stability about 2 weeks, indicating the constructed Gr-PQQ/CTS possessed good stability on GCE. The decrease of current response could be ascribed to the forming of dimers during the oxidation of NADH, leading to the fouling of electrode (Moiroux and Elving, 1978).

3.6. Biological sample analysis in human serum

The current-time method was used for quantitative determination of NADH on the optimal Gr-PQQ/CTS film modified GCE (Fig. 6). The potential was polarized at $+0.32$ V in PBS with a stirrer, which was titrated with NADH stock solution (final concentration of $10 \mu\text{M}$ for each aliquot). The Gr-PQQ/CTS film electrode responded immediately (~ 2 s) to each addition of NADH, with a sensitivity of $0.421 \mu\text{A} \mu\text{M}^{-1} \text{cm}^{-2}$. These results were

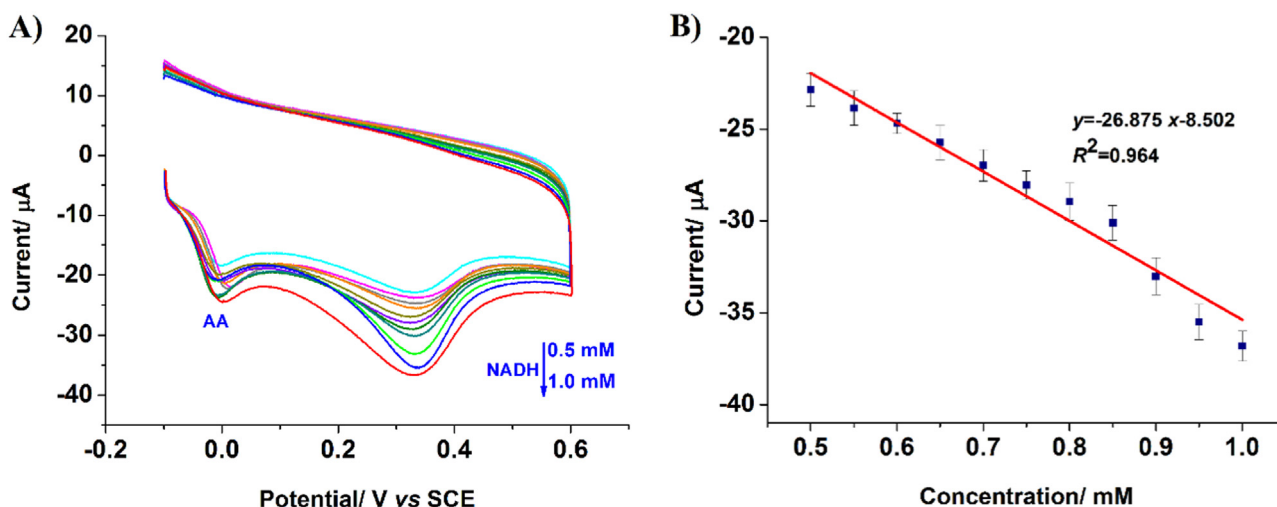


Fig. 5. (A) CV curves at Gr-PQQ/CTS film modified GCE in 0.2 M PBS (pH 7.4) containing various concentrations of NADH from 0.5 to 1.0 mM (with a fixed-interval of 0.05 mM) and AA of 0.5 mM. (B) Linear relationship between the oxidation peak currents and the concentrations of NADH.

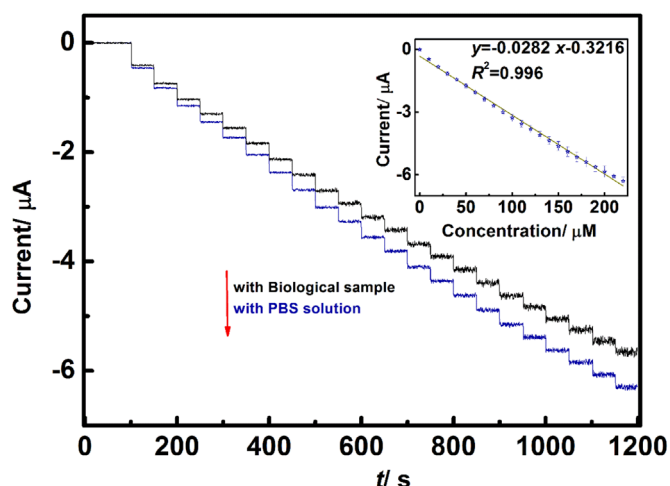


Fig. 6. Amperometric current–time response to the increasing NADH concentration (10 μM each step) at Gr-PQQ film-modified GCE in PBS (0.2 M, pH 7.4). The applied potential is +0.32 V and the stirring rate is 500 rpm. Inset: The corresponding calibration curve of NADH range from 0 to 220 μM on Gr-PQQ/CTS modified GCE.

much better than the gold nanoparticles coated reduced GO/ GCE (Chang et al., 2011). The electrode exhibited a wide linear range from 0.32 to 220 μM ($R^2 = 0.996$) and a limit of detection (LOD) of 0.16 μM ($S/N = 3$) (Fig. 6 inset). Such a high sensitivity was further reflected by those studies reported under similar electrochemical sensing strategies (Table 1).

The Gr-PQQ/CTS film modified GCE was further investigated to

testify its capacity of detecting NADH in biological samples, employing the standard titration method. As displayed in Fig. 6, the amperometric response by addition of 10 μM NADH was about 0.3233 μA in PBS containing 1% serum samples and comparable with that of ~0.36 μA in PBS. The general recovery rate of NADH is about 89.8%, verifying that Gr-PQQ composites simultaneously produced electrochemical reduction with so strong potential that the Gr-PQQ/CTS composited can be used in biological samples analysis.

4. Conclusions

In summary, we have presented a facile and cost-efficient strategy to fabricate environmentally friendly, self-assembled Gr-PQQ/CTS composites through PQQ adsorption on Gr nano-interface. The Gr-PQQ/CTS film modified GCE exhibited fast electron transport ability and low surface resistance, with a negative potential shift of 260 mV for NADH oxidation and enhanced current response due to the synergistic effect by PQQ and Gr. This electrode provided a rapid amperometric response of the NADH electrochemical detection with high stability, high sensitivity and a low limit of detection. Besides, the Gr-PQQ/CTS modified GCE revealed a robust capability of anti-interference, accompanying with acceptable recovery in serum samples. This innovative method represented in this study not only supplies an effective and simple platform to detect NADH, but also provides a new strategy to design different pollution free Gr adsorption composites used in electrochemical sensing and energy field.

Table 1

Comparison of the various electrodes in recent reports for electrochemical detection of NADH.

Electrode materials	Sensitivity ($\mu\text{A } \mu\text{M}^{-1} \text{ cm}^{-2}$)	LOD (μM)	Response time (s)	Ref
N ₂ incorporated diamond nanowire	0.141	0.3	–	Shalini et al., 2014
Co ₃ O ₄ nanosheet	0.0276	4.25	–	Chen et al., 2013
Poly Ni(II)- Curcumin	0.23	0.18	–	Ouyang et al., 2014
Gold nanorods/silicate sol-gel	0.042	0.38	–	Jayabal and Ramaraj, 2015
CoFe ₂ O ₄ -EGO	0.032	0.38	–	Ensafi et al., 2016
Au-RGO/Chit	0.318	1.2	5	Chang et al., 2011
Gr-PQQ/CTS	0.421	0.16	2	This work

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2016.04.092>.

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