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A novel poly(cyanocobalamin) modified glassy carbon electrode as electrochemical sensor for voltammetric determination of peroxynitrite

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ABSTRACT

This report described the direct voltammetric detection of peroxynitrite ($ONOO^-$) at a novel cyanocobalamin modified glassy carbon electrode prepared by electropolymeriation method. The electrochemical behaviors of peroxynitrite at the modified electrode were studied by cyclic voltammetry. The results showed that this new electrochemical sensor exhibited an excellent electrocatalytic activity to oxidation of peroxynitrite. The mechanism of catalysis was discussed. Based on electrocatalytic oxidation of peroxynitrite at the poly(cyanocobalamin) modified electrode, peroxynitrite was sensitively detected by differential pulse voltammetry. Under optimum conditions, the anodic peak current was linear to concentration of peroxynitrite in the range of 2.0×10^{-6} to 3.0×10^{-4} mol L^{-1} with a detection limit of 1.0×10^{-7} mol L^{-1} (S/N of 3). The proposed method has been applied to determination of peroxynitrite in human serum with satisfactory results. This poly(cyanocobalamin) modified electrode showed high selectivity and sensitivity to peroxynitrite determination, which could be used in quantitative detection of peroxynitrite in vivo and in vitro.

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1. Introduction

Peroxynitrite anion (O=N–O–O^-) is a reactive nitrogen species formed in vivo by the reaction between nitric oxide ($NO^•$) and superoxide anion ($O_2^{•-}$). This diffusion-limited cellular reaction is extremely fast as well as biologically significant because it transforms these two free radicals into a much more reactive species [1]. Peroxynitrite and its derivative intermediates are involved in many reactions of oxidation and nitration, causing cellular damage such as oxidation of thiol proteins and lipids, induction of single strand DNA breaks, and nitration of DNA and amino acid residues of proteins [2–4]. As a potent oxidizing and nitrating agent, peroxynitrite formation and reactions are proposed to contribute to the pathogenesis of a series of diseases, including inflammatory processes, ischemia-reperfusion injury, cardiovascular diseases, cancer, asthma, diabetes, and neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease [1,5]. Thus, accurate detection and quantification of peroxynitrite in vivo are critically important in evaluation of the potentially injuring mechanism of peroxynitrite and clinical diagnosis at early stages of disease. However, the peroxynitrite assay is extremely difficult because of its short life span, high activity and low concentration in vivo. In recent years, many methods have been developed to detect peroxynitrite, such as UV–visible spectrometry [6], immunohistochemistry [7], electron spin resonance [8], chemiluminescence [9–11], fluorescence [12–16] and high-pressure liquid chromatography [17]. Although the above methods demonstrated wide detection range and low detection limits, the techniques could not be applied in real-time determination in vivo because of complex pretreatment, the lack of the required specificity and technical problems. For example, most of the above techniques (such as fluorescence, chemiluminescence) used to measure peroxynitrite are indirect methods based on oxidation reactions mediated by peroxynitrite that rely on the measurement of secondary species, which leads to systematic errors in peroxynitrite estimates. In order to obtain accurate information of peroxynitrite in vivo, it is necessary to set up a sensitive, selective, fast-response and direct detection method to monitor the varying concentrations of peroxynitrite in the biological systems.

Electrochemistry offers the possibility of direct, on-line and real-time measurements of important biological species, which has become sensitive and convenient method for biochemical analysis [18]. Nevertheless, to date there are few reports to describe electrochemical methods for the direct determination of peroxynitrite or its protonated form. Iwuntze et al. reported that peroxynitrite could be determined in simulated bodily fluid at a bare platinum electrode by linear scan voltammetry. They determined that this process involved the two-electron oxidation of peroxynitrite and reported a detection limit of 1.6×10^{-7} mol L^{-1} [19]. Zakharova et al. described the direct voltammetric detection of peroxynitrite at a

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mercury film electrode at alkaline pH. The voltammetric response of peroxynitrite is observed as a cathodic inverted peak when the potential is swept in the oxidative direction. They also investigated the various parameters affecting the determination of peroxynitrite at the electrode such as solution pH and scan rate [20]. Jin et al. reported an electrochemical ultramicrosensor for peroxynitrite detection in vivo using manganese tetraaminophthalocyanine (MnTAPc) modified platinum or carbon ultramicroelectrode. The detection of peroxynitrite was based on electrocatalytic reduction of peroxynitrite at poly-MnTAPc modified electrode by differential pulse amperometry [21]. Also, Kubant et al. [22] reported on the use of the manganese porphyrin based electrode. But in both cases, no calibration of the electrode was accurately performed in order to assess the performance of the sensor. Thus, Bedioui's group reported the calibration of a chemically modified Pt ultramicroelectrode by poly-MnTAPc film for the determination of peroxynitrite in aqueous solution. The sensitivity of the sensor is 14.6 nA mmol L^{-1} and its detection limit is 5 μmol L^{-1} [23]. Amatore's group provided the first true direct voltammetric determination of peroxynitrite under physiological conditions. Using platinized carbon ultramicroelectrode placed adjacent to an artificial synapse constructed by a living cell, pricked with a micropipette to simulate oxidative stress bursts, Amatore et al. were able to detect the direct one-electron oxidation of peroxynitrite in vitro under simulated physiological conditions [24]. Recently, they reported the first real-time amperometric analysis of peroxynitrite released by single immunostimulated macrophages using the platinized carbon microelectrodes [25].

Although the reported electrochemical methods have successfully used for the measurements of peroxynitrite, peroxynitrite at the conventional bare electrodes (Pt, glassy carbon and platinized carbon) yielded poor electrochemical responses, and the bare electrodes exhibited low sensitivity, selectivity and reproducibility. Chemically modified electrodes (CMEs) have received increasing attentions in the past decades, which enhance the sensitivity, selectivity and reproducibility of electrochemical analysis techniques. As yet, there are almost no reports to describe CMEs for measurement of peroxynitrite except for poly-MnTAPc modified Pt ultramicroelectrode [21–23]. In this paper, we prepared a novel modified electrode with electropolymerized film of cyanocobalamin to catalyze the peroxynitrite oxidation, which provided a simple and sensitive voltammetric method for determining peroxynitrite concentrations.

Vitamin B_{12} is the name for a whole class of chemicals with B_{12} activity, which can help in the formation of red blood cells and aid the function of the nervous system. Vitamin B_{12} derivatives with common structure of naturally cobalt corrin have been shown to have similar catalytic activity as cobalt tetrasulphophthalocyanine [26]. Electrodes modified with vitamin B_{12} derivatives have been shown to catalyze many reactions including the reduction of dissolved oxygen [27], alkyl halides [28], reductive organic cyclizations [29], the oxidation of L-cysteine [30], glutathione [31], hydrazine [32] and the oxidation and reduction of nitric oxide [33]. However, the reported CMEs were modified with VB_{12} derivatives by adsorption method or drop-coating technique, which could affect the life span and the reproducibility of modified electrode because of the instability of the adsorption layer. Cyanocobalamin is the most common form of the vitamin B_{12} family under which cobalamin is extracted from living organisms, and is possibly involved in cyanide metabolism. In this work, we investigated the preparation and basic electrochemical behavior of poly(cyanocobalamin) modified glassy carbon electrode (GCE). The experimental results indicated that poly(cyanocobalamin) film had electrocatalytic activity to the oxidation of peroxynitrite. Moreover, the modified electrode showed good sensitivity, selectivity and reproducibility. Based on its excellent characteristics compared to other reported electrochemical methods in terms of high sensitivity, wide linearity and good stability, this new electrochemical sensor based on poly(cyanocobalamin) film was satisfactorily used for the quantitative determination of peroxynitrite in human serum by differential pulse voltammetry (DPV).

2. Experimental

2.1. Chemicals

Cyanocobalamin, ascorbic acid, glucose, L-arginine, L-glycine and uric acid were purchased from Shanghai Chemical Reagent Co., Ltd. (Shanghai, China). Dopamine, xanthine, glutathione, hypoxanthine and albumin were obtained from Sigma (USA). All chemicals were of analytical grade and were used without further purification. The 0.1 mol L^{-1} phosphate buffer solutions with various pH values were prepared by mixing the stock solutions of 0.1 mol L^{-1} KH_2PO_4 and K_2HPO_4. The working solutions of peroxynitrite were prepared just prior to use. Doubly distilled water was used throughout.

2.2. Synthesis of peroxynitrite

Peroxynitrite was synthesized according to previously reported procedures [34]: A 25.00 mL solution containing 1.00 g sodium nitrite was rapidly mixed with an equal volume of aqueous solution containing 1.50 mL of 35% hydrogen peroxide and 0.40 mL of 96% sulphuric acid. The resulting mixture was immediately quenched with the same volume of 1.5 mol L^{-1} NaOH. Then the excess H_2O_2 was removed by adding 0.40 g MnO_2 powder. The product was filtrated to eliminate the excess MnO_2 and stored at $-18\,^{\circ}C$. Peroxynitrite concentration was determined by UV spectrometry at 302.0 nm (ε = 1670 L mol^{-1} cm^{-1})[13].

2.3. Apparatus

Electrochemical measurements were performed with a LK2005 Microcomputer-based electrochemical system (LANLIKE, Tianjin, China). A conventional three-electrode cell was used, including a saturated calomel electrode (SCE) as reference electrode, a platinum sheet electrode as the counter electrode and a bare or modified glassy carbon disk electrode (GCE) with a diameter of 4 mm used as working electrode. All pH measurements were made with a pHS-3C digital pH meter (Shanghai Lei Ci Device Works, Shanghai, China) with a combined glass electrode. A KQ-250B ultrasonic washer (Kunshan Ultrasonic Instrument Works, Kunshan, China) was used to wash the electrode.

2.4. Preparation of the poly(cyanocobalamin) modified electrode

Cyclic voltammetry (CV) was used to form polymerization film. Prior to its modification, the bare GCE was polished with 0.05 μm α-alumina powder and rinsed with 1:1 HNO_3 solution, ethanol, and doubly distilled water for 10 min successively. After the electrode was pretreated electrochemically by scanning in a 0.5 mol L^{-1} H_2SO_4 solution between -0.4 and 1.5 V at 100 mV s^{-1} for 10 cycles, the cyanocobalamin modified electrode was prepared by electropolymerization. The polymertic film was deposited by cyclic sweeping from -1.2 to 1.0 V at 50 mV s^{-1} for 20 cycles in a dimethylformamide (DMF) solution containing 2.0×10^{-3} mol L^{-1} of cyanocobalamin and 0.1 mol L^{-1} sodium perchlorate.

2.5. Experimental methods

Cyclic voltammetric and differential pulse voltammetric measurements were carried out with three electrodes in phosphate buffer solution. The cyclic voltammograms were recorded by

cycling the potential between −0.4 and +1.4 V at a scan rate of 50 mV s^{-1}. The differential pulse voltammetric measurements were performed by applying a sweep potential from +0.2 to +1.2 V at pulse amplitude of 50 mV and pulse width of 0.1 s. Before any electrochemical experiment was performed, pure nitrogen was purged in the solution for 5 min to remove dissolved oxygen from the solution. All experiments were carried out at room temperature. The poly(cyanocobalamin) modified electrode can be used repeatedly after rinsed with doubly distilled water and blotted with filter paper.

2.6. Sample preparation

Blood samples were collected from healthy volunteers at the Hospital of Shandong Normal University. About 10 mL fresh blood sample obtained was centrifuged at 3000 rpm for 20 min to remove all precipitating materials. The separated serum can be stored frozen until they are analyzed. The spiked serum samples were prepared as follows: 1.0 mL of serum was transferred to a color comparison tube containing 8.0 mL pH 9.2 phosphate buffer solution. After spiked with different quantities of peroxynitrite, the mixture solution obtained was diluted to 10 mL with pH 9.2 phosphate buffer. The blank serum sample was prepared by diluting 1.0 mL of serum to 10 mL with pH 9.2 phosphate buffer. Then the blank and spiked serum samples were placed in the electrochemical cell to detect peroxynitrite by the proposed DPV method, respectively.

3. Results and discussion

3.1. Preparation and characterization of electropolymerized cyanocobalamin film at the GCE surface

Cyclic voltammetry was used to form the electropolymerization film. The effect of various parameters on the polymer growth such as potential scan range, solvents and supporting electrolytes was studied. The potential scan range was the most important factor in preparing poly(cyanocobalamin) film. If the positive potential value for polymerization was below 0.8 V or if the negative one was above −1.0 V, no polymer reaction occurred. The experimental result showed that the polymeric film formed was more conductive when the potential scan window was from −1.2 to 1.0 V. Therefore, it was selected as the electropolymerization potential window in this paper. Moreover, no polymer reaction occurred in the aqueous solution containing cyanocobalamin and phosphate buffer solution. Thus DMF was chose as solvent for electropolymerization. It was found that supporting electrolytes had no effect on electrodeposition process of polymertic film when 0.1 mol L^{-1} sodium perchlorate and 0.1 mol L^{-1} tetrabutylammonium tetrafluoroborate ($TBABF_4$) were used as supporting electrolyte in DMF solution, respectively. So sodium perchlorate was selected as supporting electrolyte because it is inexpensive and easy to get.

The consecutive cyclic voltammograms obtained at bare glassy carbon electrode in DMF solution containing 2.0×10^{-3} mol L^{-1} of cyanocobalamin and 0.1 mol L^{-1} sodium perchlorate were shown in Fig. 1. In the first cycle, one strong reduction peak was observed at −0.856 V (peak A), which might be the reduction of the monomer. From the second cycle on, two obvious oxidation peaks appeared with potential at −0.538 V (peak B) and 0.078 V (peak C). In the subsequent cycles the larger peaks were observed upon continuous scanning, which was reflecting the continuous growth of the film. The growth of the film was observed by monitoring the increase in the charge attributed to the redox couple of the metal centre Co(II)/(I) located at −0.85 V [35]. It could be observed that the film growth was faster for the first five cycles than for the other cycles.

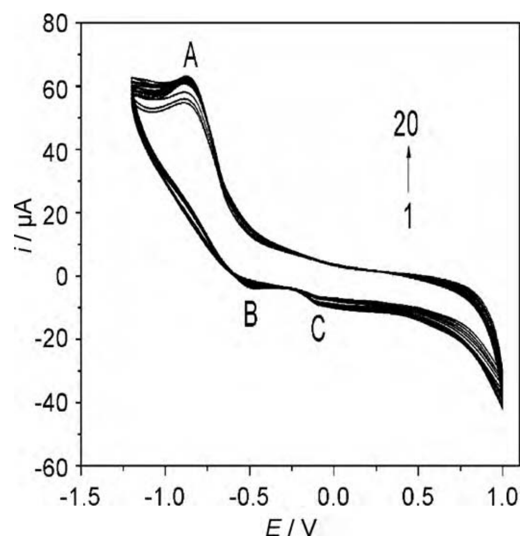


Fig. 1. Cyclic voltammograms of cyanocobalamin in electropolymerization process from 1 to 20 cycles. Cyanocobalamin: 2.0×10^{-3} mol L^{-1} in DMF solution; supporting electrolyte: 0.1 mol L^{-1} sodium perchlorate; scan rate: 50 mV s^{-1}.

From the nineteenth cycle on, the film hardly grew, which showed that polymerization had reached saturation. So the total number of the electropolymerizing scans was selected as twenty for forming stable polymer film with low electrical resistance. The shape of the voltammetric feature was that of a surface-immobilized redox couple, suggesting the formation of a redox-active polymer film on the electrode surface [21]. After modification, a uniform adherent dark red polymer was formed on the GCE surface. The above facts demonstrated that cyanocobalamin was deposited on the surface of GCE by electropolymerization.

The electrochemical behavior of the poly(cyanocobalamin) modified electrode was studied in pH 7.0 phosphate buffer solution by CV. The voltammogram in Fig. 2 showed two reversible electron transfer processes in the moderate scan rate of 100 mV s^{-1}, in which the well-defined pair of peaks located at ca. 0.05 V (peaks I) and −0.80 V (peaks II) were assigned to the redox process of Co(III)/(II) and Co(II)/(I) couples of modified electrode respectively [30]. The amplitudes of these two redox peaks varied linearly with the scan rate, which displayed surface wave characteristics. This result indicated that cyanocobalamin was deposited on the surface of GCE and the central metal Co in the polymer film had good redox activity. The modified electrode exhibited a high stability whether it was placed in dry state or in pH 7.0 phosphate buffer. No loss of elec-

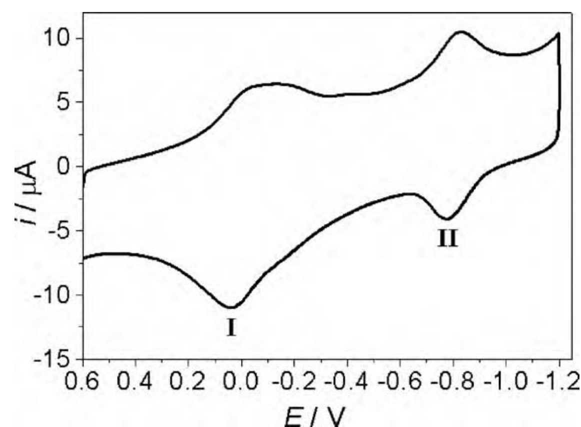


Fig. 2. Cyclic voltammogram of poly(cyanocobalamin) modified glassy carbon electrode in phosphate buffer solution. Scan rate: 100 mV s^{-1}; pH 7.0.

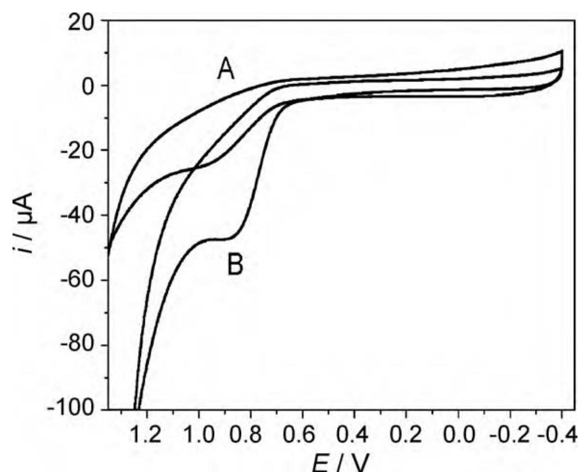


Fig. 3. Cyclic voltammograms of peroxynitrite at bare glassy carbon electrode (A) and cyanocobalamin modified electrode (B). Peroxynitrite: 1.0×10^{-4} mol L^{-1}, scan rate: 50 mV s^{-1}, pH 9.2.

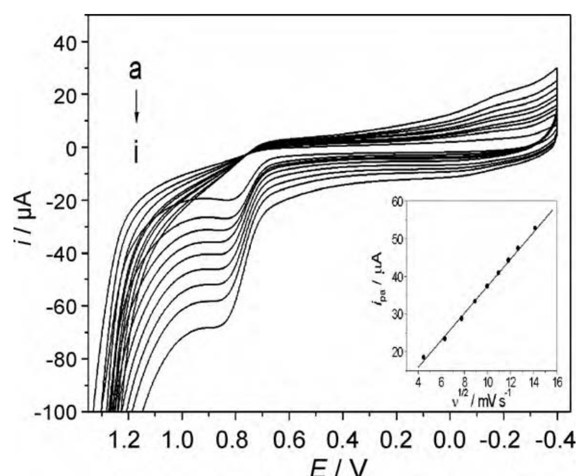


Fig. 4. Cyclic voltammograms of peroxynitrite at modified electrode at different scan rates. (a) 20, (b) 40, (c) 60, (d) 80, (e) 100, (f) 120, (g) 140, (h) 160, (i) 200 mV s^{-1}; peroxynitrite: 1.0×10^{-5} mol L^{-1}; pH 9.2. The inset shows the plot of dependence of i_{pa} on $\nu^{1/2}$.

troactivity of the electrode was found for the continuously cyclical sweeping for 6 h. The relative lifetime of the modified electrode was evaluated by periodically measuring the response sensitivity of peroxynitrite (2.0×10^{-5} mol L^{-1}) by differential pulse voltammetry. If the electrode lost 10% of the initial signals, they were considered disabled. When the poly(cyanocobalamin) modified electrode was kept at room temperature, the sensitivity remained more than 95% of the initial signal after 4 weeks storage, which indicated that the modified electrode was not deteriorated even for 1 month.

3.2. The electrochemical behaviors of peroxynitrite at poly(cyanocobalamin) modified electrode

Fig. 3 illustrated the CVs of 1.0×10^{-4} mol L^{-1} peroxynitrite at the bare electrode (curve A) and modified electrode (curve B) in pH 9.2 phosphate buffer solution. At bare GCE, peroxynitrite exhibited poor electrochemical response and no well-defined peak of its oxidation could be seen. However, a very well-defined oxidation peak appeared at 0.836 V when the modified electrode was used in the same solution. By comparison, the oxidation peak potential shifted negatively about 200 mV, and the oxidation peak current was about three times higher than that at the bare electrode. The enhanced current response and the lowered overpotential clearly indicate that poly(cyanocobalamin) film can accelerate the rate of electron transfer of peroxynitrite and have strong electrocatalytic activity towards its anodic process.

The influence of scan rate on the electrochemical response of peroxynitrite at the modified electrode was investigated by cyclic voltammetry. The oxidation peak current exhibited a linear relation to the square root of scan rate in the range between 20 and 200 mV s^{-1} with the linear regression equation of i_{pa} (μA) = 1.065 + 3.649 $\nu^{1/2}$ (mV s^{-1}) (correlation coefficient, r = 0.9990). The results in Fig. 4 indicated that the electron transfer reaction was diffusion-controlled process. In addition, it was observed that the catalytic oxidation peak potential (E_{pa}) shifted slightly to more positive potentials with increasing scan rate. The analysis of these data showed that the plot of E_{pa} vs. the logarithm of scan rate presented a linear relation (r = 0.9931), indicating that the electrocatalytic oxidation of peroxynitrite on the modified electrode surface is irreversible [36]. The slope of this straight line was equal to 40.8 mV decade^{-1}. According to the following equation, which is valid for a totally irreversible diffusion-controlled process [36],

$$E_{pa} = A + \frac{2.3RT}{2(1-\alpha)n_\alpha F}\log \nu \quad (1)$$

$$A = E^{0'} + \frac{RT}{(1-\alpha)n_\alpha F}\left[0.78 + \frac{2.3}{2}\log\left(\frac{(1-\alpha)n_\alpha FD}{k^2 RT}\right)\right] \quad (2)$$

where α is the transfer coefficient, n_α is the number of electrons involved in the rate determining step, ν is scan rate, k is the heterogeneous electron transfer rate constant, $E^{0'}$ is the formal potential, D is diffusion coefficient (for peroxynitrite, it is 1.63×10^{-5} cm^2 s^{-1} [19]) and the remaining symbols have their usual significances, we could calculate that the kinetic parameters of the electrocatalytic process $(1-\alpha)n_\alpha$ is 0.698 and k is 2.36×10^{-2} cm s^{-1}.

3.3. Effect of pH on the voltammetric response of peroxynitrite

Peroxynitrite anion (O=N–O–O$^-$) is relatively stable and long-lived under alkaline conditions, but at lower pH the protonated form, ONOOH rapidly rearranges/decomposes to form nitrate. Because peroxynitrite decomposes within less than 2 seconds at physiological pH (pH 7.4) at 37 °C, which does not apply during in vitro experiments since its decomposition kinetics are too fast at this pH, experimental conditions need to be defined so that bulk solution of peroxynitrite could be investigated without significant decomposition during the analytical measurement. The decomposition of peroxynitrite in dilute solution is highly sensitive to pH as a result of the central involvement of its transient protonated form (pK_a = 6.8). The kinetics could be considerably slowed down by performing the experiments at basic pHs [39]. Therefore, the effect of pH on the catalytic responses of peroxynitrite at the modified electrode was studied in moderately basic aqueous media, in which the peroxynitrite concentration did not reasonably evolve.

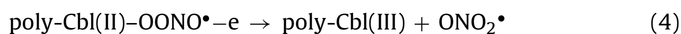
Influence of supporting electrolyte on the electrochemical oxidation signal of peroxynitrite was firstly examined in various electrolytes, including pH 9.2 borate buffer, ammonia-ammonium chloride buffer and phosphate buffer. It was found that the oxidation peak was well shaped, and the oxidation peak current was highest in pH 9.2 phosphate buffer. Therefore, phosphate buffer at basic pHs was used as supporting electrolyte. Then, the effect of pH was investigated over the pH range from 8.8 to 10.2. The results showed that the anodic peak potential (E_{pa}) remained constant (figure not shown) in this range. The average E_{pa} was 0.832 ± 0.006 V versus SCE. Moreover, the anodic peak current did not change with

increasing the pH in tested range. But the oxidation peak shape became bad at pHs ≥ 9.4, which could decrease the resolution and sensitivity of anodic peak. So pH 9.2 phosphate buffer was chosen for the determination of peroxynitrite.

3.4. Discussion of the possible mechanism for the voltammetric behavior of peroxynitrite at poly(cyanocobalamin) electrode

The absence of any systematic dependence of E_{pa} on the pH indicated that the electrochemical process does not involve protons [36]. This implies that the anodic peak represents the irreversible oxidation of ONO_2^- (viz., not of its conjugate acid), which is consistent with the fact that pH > pK_a 6.8 over the range investigated. The earlier studies have shown that cobalt (II) tetraphenylporphyrins (Co(II)TPP) can bind the O* atom in peroxynitrite (O=N–O–O*⁻) to form an axial coordination compound (Co(II) –OONO–TPP). Moreover, kinetic and mechanistic experiments have elucidated the reversible binding nature of the reaction of peroxynitrite with Co(II)TPP [38].Because cyanocob(II)alamin (Cbl(II)) also contains cobalt(II) porphyrin, we speculated that Cbl(II) can bind peroxynitrite to form a stable Cbl(II)– $OONO^-$ coordination compound. Furthermore, since the maximum oxidation degree of nitrogen is +VI as it is in ONO_2^- [37], this implies that the primary redox reaction is a one-electron process giving rise to $ONO_2^\bullet$ as the primary intermediate. Then $ONO_2^\bullet$ decomposes to form NO_2 and O_2 [24]. The following equations showed the postulated electrocatalysis mechanism of poly(cyanocobalamin) film toward peroxynitrite oxidation.

$$\text{poly-Cbl(III)} + \text{O=N–O–O}^{\bullet -} \rightarrow \text{poly-Cbl(II)–OONO}^\bullet \quad (3)$$



$$\text{poly-Cbl(II)–OONO}^\bullet - e \rightarrow \text{poly-Cbl(III)} + ONO_2^\bullet \quad (4)$$

$$2ONO_2^\bullet \rightarrow O_2 + 2NO_2 \quad (5)$$

The postulated mechanism demonstrated the redox-active cobalt centre in poly(cyanocobalamin) film can catalyze peroxynitrite decomposition through the formation of intermediates, which is in agreement with the mechanism of peroxynitrite decay in basic aqueous media proposed previously [24,39].

3.5. Determination of peroxynitrite at poly(cyanocobalamin) modified electrode

Under the optimum conditions, differential pulse voltammetry (DPV) was used to detect peroxynitrite at the modified electrode because of its higher current sensitivity and better resolution than CV. Fig. 5 illustrated the DPV responses of the modified electrode while the concentration of peroxynitrite increased. It can be seen that the anodic peak current increased linearly with increase of peroxynitrite concentration. A linear relationship can be established between i_{pa} and the concentration of peroxynitrite in the range of 2.0×10^{-6} to 3.0×10^{-4} mol L^{-1}.The linear regression equation and correlation coefficient are: i_{pa} (μA) = 0.08091C (μmol L^{-1}) + 0.2946 (r = 0.9986). The detection limit of the electrochemical sensor was defined as peroxynitrite concentration yielding a signal equal to three times the standard deviation of the background current. In the present paper, we performed six repeated determinations and obtained an average result with a calculated detection limit of 1.0×10^{-7} mol L^{-1} based on the signal-to-noise ratio of 3 (S/N). The relative standard deviation (RSD) of the determination of 6.0×10^{-5} mol L^{-1} peroxynitrite repeated for six times was calculated to be 1.98%, which demonstrated the good reproducibility of the proposed electrode.

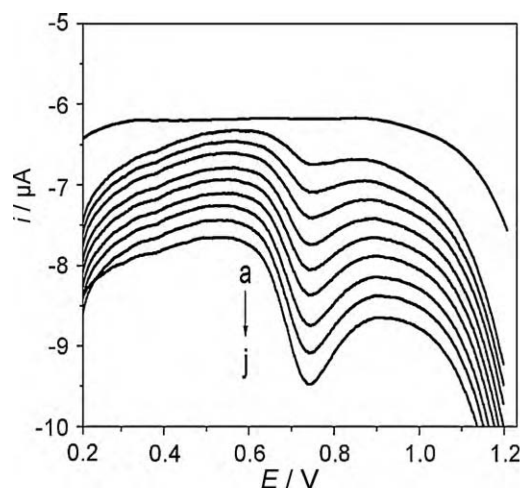


Fig. 5. Differential pulse voltammograms of different concentrations of peroxynitrite at the poly(cyanocobalamin) modified electrode. (a) 0, (b) 2.0, (c) 3.0, (d) 4.0, (e) 5.0, (f) 6.0, (g) 7.0, (h) 8.0, (i) 9.0, (j) 10.0 μmol L^{-1}; Pulse amplitude: 50 mV; pulse width: 0.1 s; pH 9.2.

The proposed method had high sensitivity, wide linear range and good stability compared with other methods. The comparison of this method with other electrochemical methods for the determination of peroxynitrite was listed in Table 1.

3.6. Interferences

For evaluating selectivity of the modified electrode, the influences of various interfering species on the determination of 2.0×10^{-5} mol L^{-1} peroxynitrite in pH 9.2 phosphate buffer solution were investigated by DPV. The interfering agents include a variety of potential interferences present in biological samples such as dopamine (DA), ascorbic acid (AA), uric acid (UA), xanthine (XA), NO and NO_2^-. The tolerance limit was taken as the maximum concentration of the foreign substances that caused an approximately ±5% relative error in the determination. The results showed that the tested coexisting interferents brought the detection signal deviation below 5% under sufferable coexisting amount, which demonstrated that the above substances have no apparent effects on the response of peroxynitrite at the modified electrode. The experimental results of interference are as follows: 100-fold of glucose, NO_3^-, L-arginine, L-glycine, 50-fold of NO_2^-, H_2O_2, AA, UA, XA and 20-fold of DA, reduced glutathione (GSH), hypoxanthine, NO and albumin did not interfere with the determination of 2.0×10^{-5} mol L^{-1} peroxynitrite. From the experiments of the interference, it seems that the poly(cyanocobalamin) modified electrode should have interference-free signals for peroxynitrite measurements in vivo because the concentrations of studied interferents in biological samples (e.g. DA at submicromolar concentration and hypoxanthine at micromolar concentration

Table 1
Comparison of the proposed method with other electrochemical methods for the determination of peroxynitrite.

Electrode	Linear range	Detection limit	Ref.
Bare platinum	1.6×10^{-4} to 1.0×10^{-3}	1.6×10^{-7}	[19]
Mercury film	1.2×10^{-5} to 2.1×10^{-4}	1.2×10^{-5}	[20]
Poly(manganese tetraaminophthalocyanine)	1.66×10^{-5} to 1.15×10^{-4}	5.0×10^{-6}	[23]
Poly(cyanocobalamin)	2.0×10^{-6} to 3.0×10^{-4}	1.0×10^{-7}	This work

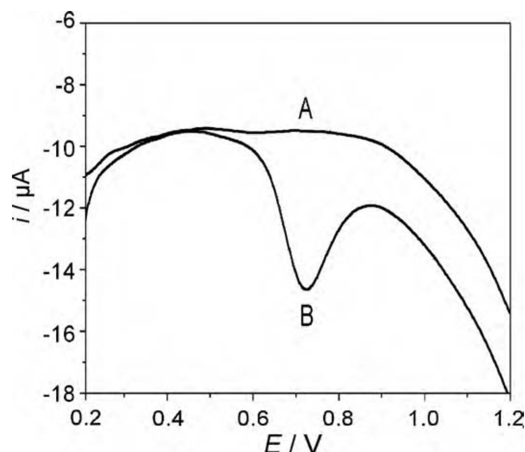


Fig. 6. Differential pulse voltammograms of peroxynitrite in human serum samples: (A) blank serum sample; (B) serum sample spiked with 40.0 μmol L^{-1} standard solution of peroxynitrite. Other conditions as in Fig. 5.

Table 2
Determination of peroxynitrite in spiked human serum samples ($n = 6$).

Sample no.	$ONOO^-$ added (μmol L^{-1})	$ONOO^-$ found (μmol L^{-1})	Recovery (%)	RSD (%)
1	4.0	3.89	97.3	2.5
2	8.0	8.16	102.0	2.3
3	40.0	40.32	100.8	1.9
4	80.0	78.85	98.6	2.2

in human serum) are far lower than sufferable coexisting amount, which can be applicable to detection of real biological samples.

3.7. Determination of peroxynitrite in biological samples

Results of voltammetric detection of peroxynitrite in spiked human serum were shown in Fig. 6. Curves A and B represent differential pulse voltammograms for blank serum sample and a spiked serum sample containing 4.0×10^{-5} mol L^{-1} peroxynitrite standard solution, respectively. It can be seen that the presence of biological substances in human serum such as proteins and amino acids do not interfere with the determination of peroxynitrite. The poly(cyanocobalamin) modified electrode can be used repeatedly for each sample analysis after rinsed with doubly distilled water and blotted with filter paper. The recovery data were shown in Table 2, which were average values of six repeated determinations. The satisfactory results demonstrated that the proposed method has a good precision. Although the cellular concentration of peroxynitrite is not exactly known, as it depends on the excess concentration of superoxide anion and nitric oxide, it is perhaps expected to be in the micromolar range [1]. It is therefore believed that the proposed method with high sensitivity and selectivity can be applied to voltammetric measurement of peroxynitrite in cell samples and other biological samples.

4. Conclusions

The novel poly(cyanocobalamin) modified electrode had been prepared and used as electrochemical sensor for the determination of peroxynitrite in alkaline pH solution by DPV. Compared with other electrochemical methods, the modified electrode reported here has some advantages, such as higher sensitivity, better stability, good biocompatibility and less likely to result in environmental pollution. The proposed method was applied to the determination of peroxynitrite in real biological samples with satisfactory results. Hence, we believe that the poly(cyanocobalamin) modified electrode can be applicable to the accurate detection and study of peroxynitrite in biological systems.

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