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An Amperometric Nitrite Biosensor Based on the Bioelectrocatalysis of Hemoglobin Incorporated in Sol-gel Film

Yixin Sun,[a,b] Shengfu Wang [b,*]

[a] School of Sciences, Zhejiang Forestry University, Lin'an 311300. P R China
[b] Ministry-of-Education Key Laboratory for the Synthesis and Application of Organic Functional Molecules & College of Chemistry and Chemical Engineering, Hubei University, Wuhan 430062, P. R. China
***Corresponding Authors:**
S. Wang
Phone: +86-27-50865309
Fax: +86-27-88663043
E-mail address: wangsf@hubu.edu.cn (S. Wang)

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Abstract

A highly sensitive, fast and stable biosensor for determination of nitrite was developed using hemoglobin immobilized on a poly (N-isopropylacyamide-co-3- methacryloxypropyltrimethoxysilane) (PNM) modified glass carbon electrodes. The matrix provided a biocompatible microenvironment for retaining the native structure and activity of the entrapped hemoglobin. Nitrite could be reduced by the electrocatalysis of the entrapped hemoglobin without any mediator. The reagentless sensor exhibited a fast response (less than 8 s) and sensitivity as high as 0.73 μA mM cm^{-2}. The linear range for nitrite determination was from 0.11 to 1.88 mM with a detection limit of 2.0×10^{-5} mol L^{-1}.

Keywords: Hemoglobin; PNM; Bioelectrocatalysis; Biosensor; Nitrite.

1. Introduction

Nitrite is one of the well-known inorganic pollutants in environmental, food, industrial and physiological systems. The widespread nitrite pollutant becomes toxic in human body and Animals [1]. So some Communities have established the maximum admissible levels of nitrite in drinking water at 0.1 mg/L, increasing the demand for sufficiently sensitive, accurate and uncomplicated analytical procedures for nitrite. Spectrophotometric methods for the determination of nitrite have been developed over the past several decades [2-4]. However, these methods required large and expensive instrument and extensive pre-treatment of the sample and thus were limited. Biosensors incorporating proteins or enzymes have also been used for nitrite analysis in real samples with reasonable sensitivity and selectivity. Such as hemoglobin entrapped in carbonized titania nanotubes [5], Fe_3O_4 nanoparticles [6], zeolite particles [7], nitrite

reductase immobilized on Methyl viologen/ Nafion composite film [8], ordered mesoporous titanium oxide matrix [9] and so on. The development of optical nitrite biosensors is of great interest.

PNM is a kind of new sol-gel material [10], which is promising as an immobilization matrix because of its water-solubility, biocompatibility, and good film forming ability at extra-mild conditions. Herein, we studied the direct electron transfer of hemoglobin (Hb) entrapped in PNM films at glassy carbon (GC) electrodes. The immobilized Hb showed an enhanced electron transfer behavior. We found that nitrite (NO_2^-) can be catalytically reduced at the Hb-PNM/GC electrode. The simple process constructs a high-performance nitrite biosensor.

2. Experimental

Reagents. Cattle hemoglobin was from Fluka. Sodium nitrite ($NaNO_2$) was from Beijing Shuanghuan Chemicals. All other chemicals were reagent grade. All of the chemicals were used as received. All the water used in the experiment was deionized water that was purified twice successively by ion exchange and distillation. Buffers for voltammetry all contained 100 mM KBr. Buffer solution were 25 mM citrate for pH 3.0-6.0, 25 mM phosphate for pH 7.0-8.0, and 25 mM borate for pH 9.0-10.0.

Apparatus. Electrochemical measurements were performed at a CHI660 electrochemistry workstation (CH Instruments Co., USA). The electrochemical cell consisted of a three-electrode system where the modified glass carbon electrode (d = 3 mm) was used as the working electrode, a platinum wire as a counter electrode and a saturated calomel electrode (SCE) as the reference electrode. All measurements were carried out at a room temperature. All experimental solutions were deaerated by bubbling highly pure nitrogen for 15 min, and a nitrogen atmosphere was kept over the solution during measurements.

Electrode modification. Prior to coating, GC electrodes were polished with 0.05μm alumina and sonicated in deionized water three times. They were then treated in 1:1 (v/v) aqueous nitric acid for 10 min, followed by rinsing and sonication in water and methanol successively. A PNM solution (0.1 g mL^{-1}) was prepared by dissolving the PNM in a aqueous solution at refrigerator (4 ^oC) for 30 min. The concentration of the Hb stock solutions, prepared by dissolving Hb in 0.05M phosphate buffer solution (pH 7.0), was 10 g L^{-1}. A 15 μL volume of PNM and 10 μL of DMF were mixed with 15 μL of the Hb stock solution, and then 20 μL of the mixture was pipetted onto the surface of the pretreated GC and spread gently over the entire surface. A small bottle was fit tightly over the electrode so that water evaporated slowly and more uniform films were formed. The films were then dried in air overnight. The PNM film modified GC electrode was prepared in the same way as described above but without Hb.

3. Results and discussions

UV-vis spectroscopy of Hb in a PNM film. The locations of the Soret absorption band of iron heme may provide information about the denaturation of heme proteins. When heme proteins were denatured, the Soret band shifted or disappeared [11]. UV-vis spectroscopy was thus here to detect the change of Soret band of Hb in PNM films cast on the glass slides. In this study, the Hb concentration and the ratio of Hb to PNM and DMF were the same for both UV-vis spectroscopy and electrochemical experiments. Figure 1 show Soret bands of dry films cast from Hb and Hb-PNM and Hb-PNM+DMF solutions at 412, 414, 418 nm (Figure 1a, b and c), respectively. These suggest that Hb in dry-PNM films has a secondary structure nearly the same as the native state of Hb in its dry films alone and no significant denaturation has occurred for Hb-PNM films containing DMF.

The Soret band caused by visible absorbance by the Fe (III) heme in protein was sensitive to pH [12]. It can be seen that the Hb-PNM film absorption spectrum exhibits a strong heme Soret band at nearly 412 nm in the range of pH 5.0 ~ 9.0, However, when pH was decreased to below 4.0, the 412 nm Soret band absorption shifted and became broader and smaller (Fig 1d), indicative of a great change in heme pocket. These results are in agreement with those of Palaniappan [13] and Liu [14].

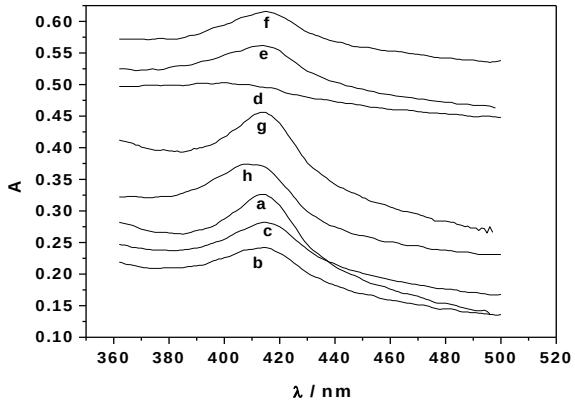

Figure 1. UV-Vis absorption spectra of Hb and Hb-PNM films on glass slides for: (a) dry Hb film; (b) dry Hb-PNM film; (c) dry Hb-PNM+DMF film and Hb-PNM films in different pH buffer solution: (d) pH 3.0; (e) pH 4.5; (f) pH 5.5; (g) pH 7.0; (h) pH 9.0

Direct electrochemistry of Hb in a PNM film. Figure 2A shows the cyclic voltammograms of Hb-PNM modified GC electrode in pH 7.0 buffer solution. It gives a pair of very stable and well-defined peaks. The peaks are located at the potentials characteristic of the heme Fe^{III}/ Fe^{II} redox couples of the proteins [15]. No redox peaks are observed at PNM modified GC electrode in the same potential range (Figure2A (a)).

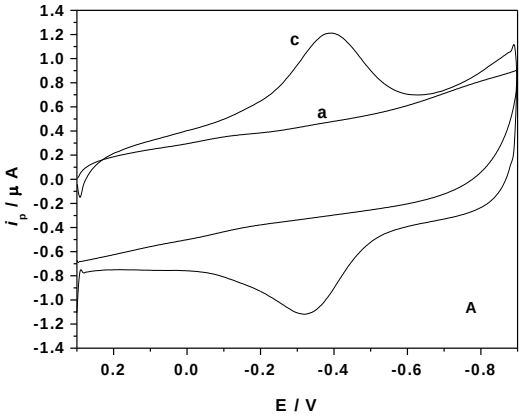

Figure. 2A: Cyclic voltammograms of PNM /GC (a) and Hb-PNM/GC(c) in pH 7.0 buffer solution at 0.2 V/s scan rates.

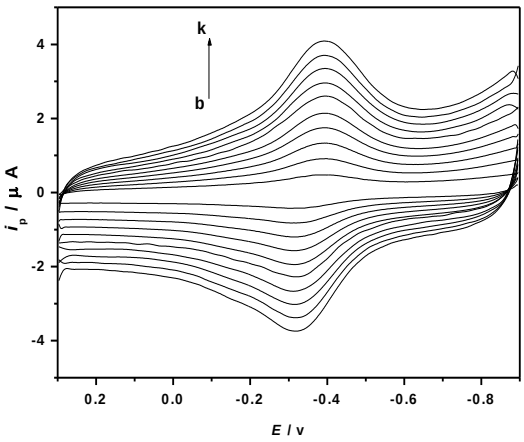

Figure 2B: Cyclic voltammograms of Hb-PNM/GC(c) in pH 7.0 buffer solution at varying scan rates (b→ k):0. 1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1V/s.

These results coincide well with previous studies on Hb immobilized in other films [16, 17]. The anodic and cathodic peak potentials of Hb-PNM/GC electrode at the scan rate $0.2Vs^{-1}$ are -0.318 V and - 0.392 V (Figure 2A(c)) respectively. The difference between the anodic peak potential (E_{pa}) and the cathodic peak potential (E_{pc})) of 74mV show that the redox process is a typical quasi-reversible electro-chemical process involving an active substance attached to the electrode. The anodic and cathodic peak currents for immobilized Hb are found to increase linearly with scan rates from 0.1 to 1 V s^{-1} (Figure 2B), as expected for thin-layer electrochemical behavior [18]. The surface coverage (Γ) of 2.07 $\times 10^{-11}$ mol cm^{-2} is estimated according to Laviron's equation [19]

$$I_p = n^2F^2 A\Gamma v/4RT.$$

This value is below the total amount of Hb deposited on the electrode surface and suggests that only those proteins in the inner layer of the films close to the electrode and with a suitable orientation can exchange electrons with the electrode.

Average formal potentials ($E^{0'}$ = −0.343 V), apparent coverage (Γ =1.56×10^{-11} mol cm^{-2}), electron transfer coefficient (α=0.50), and apparent heterogeneous electron transfer rate constants (k_s=16) were also estimated by nonlinear regression analysis of square wave voltammetry

(SWV) forward and reverse curves (Figure 3) [20].

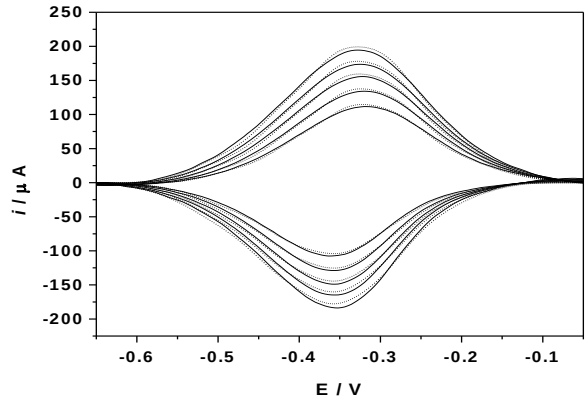

Figure 3. Square wave forward and reverse current voltammograms for Hb-PNM films in pH 7.0 buffer solution at different frequencies. The solid lines represent the experimental SWV from which background has been subtracted. Points are the best fit by nonlinear regression onto the 5- $E^{0'}$ dispersion model. SWV conditions: pulse height 60mV and frequencies (Hz): (a) 132, (b) 152, (c) 172, (d) 185.

Influence of pH on direct electrochemistry of Hb. An increase of pH of the solution leads to a negative shift in potential for both cathodic and anodic CV peaks for Hb-PNM film. As shown in Figure 4A, stable and well-defined CVs can be always obtained in the range of pH 3.0~10.0. The standard electrode potential ($E^{0'}$) for Hb in a PNM film shifts to negative with increasing pH, $E^{0'}$ has a linear relationship with linear regression equation of $E^{0'}$= 0.0784 - 0.0402 pH (R = 0.9970) for Hb (Figure 4B), indicating the electron transfer of Fe^{III}/ Fe^{II} redox couple company with the transfer of proton. All changes in CV peak potentials and currents with pH are reversible.

Electrocatalytic reduction of NO_2^- on the Hb-PNM/GC electrodes. Figure 5 shows the cyclic voltammograms of Hb-PNM/GC electrodes in pH 7.0 buffer solution. A new reduction peak appears at about - 0.82 V while the Hb Fe^{III}/ Fe^{II} peak pair is nearly intact. However, direct reduction of NO_2^- at blank film electrodes is not observed (Figure 5a), indicating that Hb entrapped in PNM film catalyze the reduction of NO_2^-.

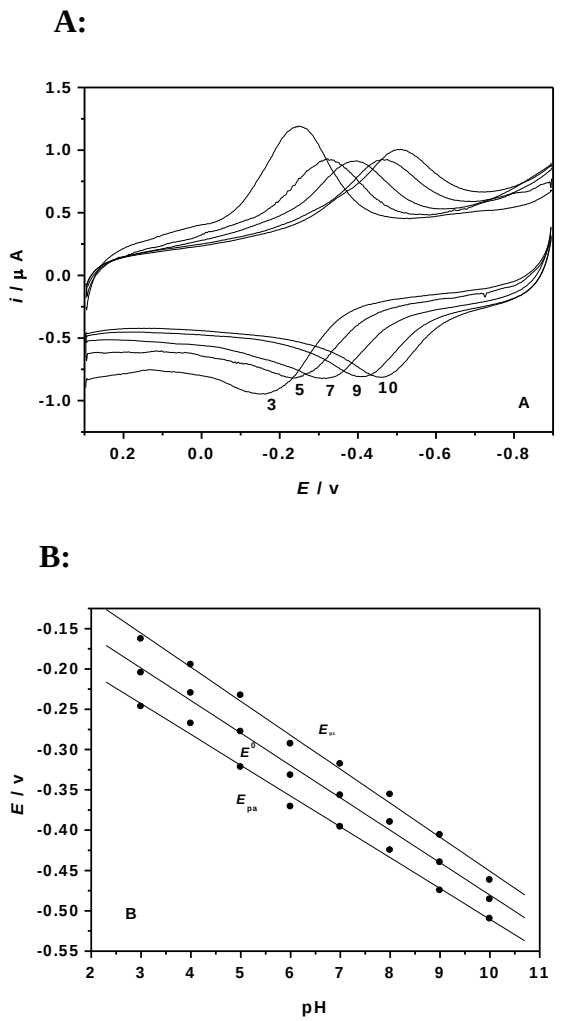

Figure 4. A: Cyclic voltammograms of Hb-PNM /GC in 0.1M buffer solution at different pHs. Scan rate: 0.2 V s^{-1}. **B:** Plots of E vs. pH.

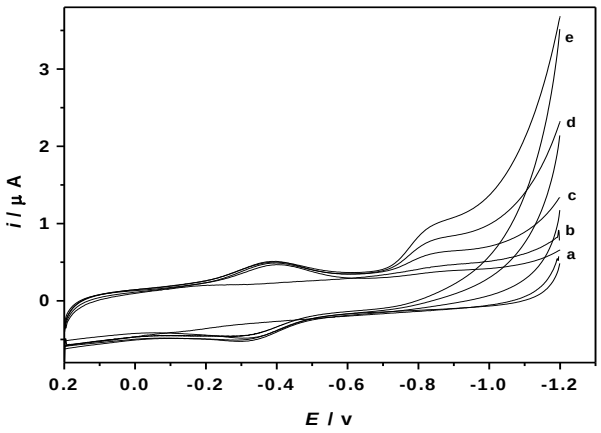

Figure 5. Cyclic voltammograms at 0.1Vs^{-1} in pH 7.0 buffer solution: (a) PNM modified GC in

buffer solution containing 6.68×10^{-4}molL^{-1} $NaNO_2$; (b) Hb- PNM films modified GC in buffer solution without $NaNO_2$; (c-e) Hb-PNM films modified GC electrode in buffer solution containing 0.167, 0.668, 1.67mM.

Figure 6 shows the amperometric response of the Hb-PM/GC electrode with successive of NO_2^- to pH 7.0 buffer solution at an applied potential of - 0.82 V. Upon addition of an aliquot of $NaNO_2$ to the buffer solution, the reduction current of NO_2^- increases steeply to reach a stable value. The sensor can achieve a steady state current within 8 s; such a short response time further proves that the PNM is a promising material for the biosensor fabrication. With an increase of NO_2^- concentration, the amperometric response increases. Inset in Figure 6 shows the calibration curve of the Hb-PNM/GC electrode to NO_2^-. The linear response range of the sensor to NO_2^- concentration is from 0.11 to 1.88 mM with a correlation coefficient of 0.9987 (n =17). The detection limit is 2.0×10^{-5} mol L^{-1} at a signal to noise ratio of 3, which was much lower than that reported for the detection limit of 0.1mM NO_2^- [8], and the sensitivity of the biosensor to NO_2^- can be calculated to be 0.73 μA mM cm^{-2}.

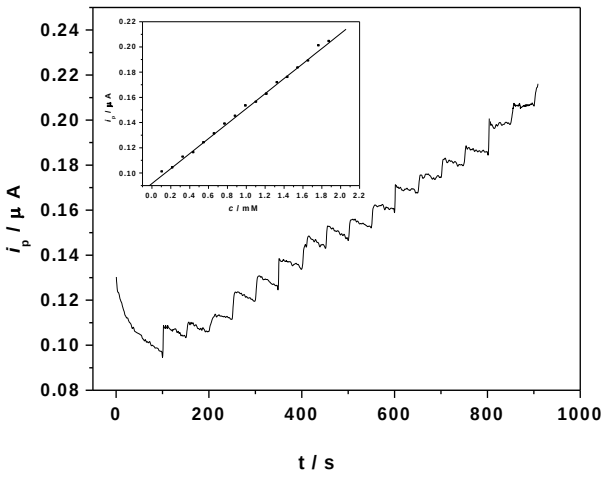

Figure 6. Typical current–time response curve of the sensor upon successive additions of 10 μL every time 0.133 mol L^{-1} NO_2^- to 12.00 mL pH 7.0 buffer solution at - 0.82 V. Inset: plot of catalytic current vs. NO_2^- concentration.

Effect of temperature on the NO_2^- biosensors. Figure 7 shows the effect of temperature on the sensitivity of the same biosensor. Like the majority of enzymes or proteins, the activity of Hb is related to temperature, With temperature increasing from 15 to 60 ^oC, the amperometric responses and the electrocatalytic activities of the immobilized Hb to NO_2^- increase. The Hb has activity even at 60 ^oC. It is evident that the immobilized Hb has good thermal stability because of the unchangeability of microenvironment and its native structure upon temperature change. These results indicate that the sensors can handle a wide range of temperature.

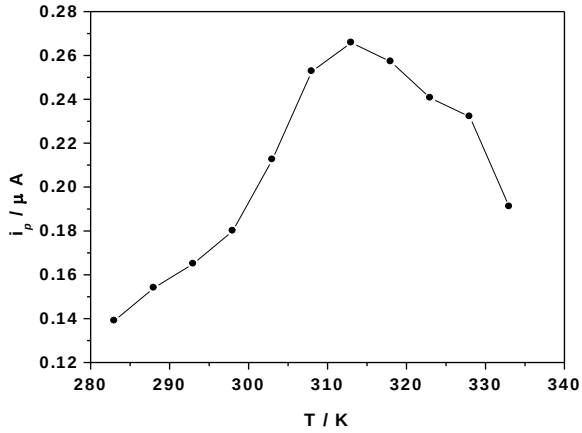

Figure 7. Effect of temperature on the response of the 5.0×10^{-4} mol L^{-1} NO_2^- biosensor.

Stability and Repeatability of the Hb-PNM/GC modified electrode. Long-term storage stability is one of the key factors of a biosensor performance. The measurement repeatability of the Hb -PNM/GC electrode was examined at a concentration of 5.0×10^{-4} mol L^{-1} NO_2^- with the same modified electrode and the relative standard deviation (RSD) was 4.3 % for nine successive assays. The fabrication of 4 electrodes, made independently, showed an acceptable reproducibility with a RSD of 4.6 % for the current determined at a NO_2^- concentration of 5.0×10^{-4} mol L^{-1}, the Hb -PNM/GC electrode were stored in 50 mM pH 7.0 buffer solution at 4 ^oC, the biosensor showed googd stability over 1 month. Thus, PNM were very efficient in retaining the electrocatalytic activity of Hb and preventing

it from leaking out of the sensor. This good stability is mainly attributed to the biocompatible of PNM and the mild immobilization process of HRP. The processes of electrode modification avoid the shortcomings caused by acidic catalyst or calcinations step needed in traditional sol-gel process and maintain their biological activity to a large extent.

4. Conclusions

A novel and simple biosensor have been successfully developed. Hb is immobilized on the PNM film and shows a good direct electrochemistry. The immobilized Hb displays a high affinity and high response sensitivity to nitrite without mediator. The sensor shows a good reproducibility and stability. PNM provides an efficient strategy and a new promising platform for the study of electron transfer of proteins and the development of biosensors.

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