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Nitrite reduction and detection at a carbon paste electrode containing hemoglobin and colloidal gold

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A novel renewable reagentless nitrite biosensor based on the direct electron transfer of hemoglobin (Hb) and a new sensing mechanism was proposed by combining the advantageous features of colloidal gold nanoparticle and carbon paste technology. The direct electrochemistry of immobilized Hb displayed a pair of redox peaks with a formal potential of −42 mV (*vs.* NHE) in 0.2 mol dm^{-3} NaAc–HAc buffer (pH 5.5). The immobilized Hb displayed an excellent response to the reduction of NO_2^- with one interfacial charge transfer followed by a chemical reaction (EC) mechanism. Under optimal conditions, the interfacial EC process could be used for the sensitive determination of NO_2^- with a linear range from 0.1 to 9.7 μmol dm^{-3} and a detection limit of 0.06 μmol dm^{-3} at 3σ. The amperometric determination of high concentrations of NO_2^- based on the irreversible reduction of NO could be performed at pH 4.0 with a linear range from 0.1 to 1.2 mmol dm^{-3}. The surface of biosensor could be renewed quickly and reproducibly by a simple polish step. The biosensor has been used satisfactorily for nitrite determination in native water samples.

Introduction

Nitrite is an important precursor in the formation of N-nitrosamines, many of which have been shown as potent carcinogens in human bodies.[1,2] It exists widely in the environment, beverages, and food products as a preservative.[3] Therefore, the importance of improved analytical methods for its detection in food, water and biological fluids has received considerable attention.[4–16] Many methods have been developed for this purpose by various techniques, such as spectrophotometry,[4,5] chromatography,[6,7] capillary electrophoresis,[8] chemiluminescence,[9] and electrochemistry.[10–16] The potential low cost and portability of electrochemical devices provide a number of attractive options. The electrochemical sensors based on metalloporphyrins,[11] ruthenium polymer,[12] nitrite reductase enzymes[13,14]and noble-metal-substituted polyoxometalates[15] have been used for the determination of nitrite in egg,[3] saliva,[12] waste water[16–18] and pickled vegetable water.[19] These sensors are favorable for nitrite determination with high sensitivity. Most of them have relatively good selectivity and a fast response. Davis and Compton presented a sonoelectrochemical approach to enhance electrode sensitivity and to improve its reproducibility for nitrite determination.[3] In this work we develop a novel renewable reagentless nitrite sensor based on the direct electron transfer of hemoglobin (Hb) and nitrite reduction. The stability of the sensor is improved greatly by combining the advantageous features of colloidal gold nanoparticle and carbon paste technology.

Hemoglobin is an important respiratory protein in red blood cells and an ideal model molecule for the study of electron transfer reactions of heme proteins. Its electrochemical behavior has been extensively studied because of its commercial availability and moderate cost.[20–31] These works provided a significant understanding of electron transfer mechanism between proteins and electrodes.[22–30] The direct electron transfer between Hb and electrodes has been achieved by incorporating Hb in polyacrylamide hydrogel film,[22] surfactant film,[23–26] clay,[28,29] SP Sephadex[30] and a DNA[32] modified membrane. These materials provided biomembrane-like microenvironments[23] and facilitated direct, chemically reversible, electron exchange between Hb and electrodes, and thus eliminated the need for mediators.[25,26]

Recently there has been an increasing interest in the electrochemical behavior and application of nanomaterials.[33] It has been demonstrated that colloidal gold, a well-known nanomaterial, can also provide a microenvironment similar to that of redox proteins in native systems and retain the biological activity of proteins upon adsorption.[34–37] Therefore, it has been used for the study of direct electron transfer of redox proteins and the preparation of hydrogen peroxide sensors.[36,37] In this work, 24 nm diameter colloidal Au particles are mixed with carbon paste to prepare a novel renewable reagentless nitrite sensor. Upon addition of NO_2^- to the supporting electrolyte, both the cathodic and anodic peak currents of hemoglobin increase, which suggests one new sensing mechanism based on the charge transfer for both NO_2^- and hemoglobin reduction followed by an irreversible chemical reaction for the formation of the complex of Hb with nitric oxide (NO).[38,39] Based on this sensing mechanism, a novel biosensor is developed for the sensitive determination of NO_2^- with the detection limit of 0.06 μmol dm^{-3} at 3σ. This biosensor can be renewed by a simple polish step and has been used for the determination of NO_2^- in real samples.

Experimental

Chemicals

Hemoglobin was obtained from Sigma and used without further purification. $HAuCl_4 \cdot 3H_2O$ was purchased from Aldrich. Other reagents were of analytical reagent grade. All solutions were prepared with double-distilled water. 0.2 mol dm^{-3} acetate buffer solutions (NaAc–HAc) of various pHs were prepared by mixing two stock solutions of 0.2 mol dm^{-3} HAc and NaAc. Standard solutions of sodium nitrite were prepared daily in a 0.2 mol dm^{-3} pH 4.0 NaAc–HAc buffer.

Carbon graphite powder (< 325 mesh, Johnson Matthey) and paraffin oil (from Fluka) were used to prepare the carbon paste. Colloidal gold was prepared according to literature[34,35] by adding 0.5 mL of a 1% Na_3citrate solution to a boiling 50 mL solution of 0.01% $HAuCl_4$. All glassware used in this procedure was cleaned in the freshly prepared 3:1 HNO_3–HCl, rinsed thoroughly in H_2O and dried prior to use. The mixture was maintained at boiling point for 15 min and stirred for another 15 min after removing the heating source to produce colloidal gold. The diameter of the colloidal gold nanoparticles was measured to be 24 ± 2.1 nm using transmission electron microscopy.[36] The preparation was stored in a brown glass bottle at 4 °C.

Electrode preparation

The carbon graphite powder was treated at 700 °C for 30 s, and then mixed with paraffin oil (1 mg : 0.36 μL) to prepare the carbon paste (CP). The colloidal gold modified CP (Au–CP) was obtained by adding colloidal gold solution to treated carbon graphite powder (2 μL : 1 mg), and then mixing paraffin oil to the mixture (1 mg : 0.36 μL) after evaporation of water for 3 h in air. The hemoglobin-Au–CP (Hb–Au–CP) was prepared by mixing hemoglobin to the above colloidal gold modified CP (1 mg : 0.45 mg). As a comparison, the Hb modified CP (Hb–CP) was prepared by thoroughly mixing Hb to CP (1 mg : 0.45 mg). A portion of these resulting pastes was packed into the end of glass tubes with an inner diameter of (0.51 ± 0.01) mm to form the different electrodes (CPE, Au–CPE, Hb–Au–CPE and Hb–CPE). Electrical contact to the paste was established by inserting a copper wire down the tubes and into the back of the mixture. These electrodes were stored at 4 °C. Prior to use the electrode tip was gently rubbed on a fine piece of paper to produce a flat surface.

Electrochemical measurements

Electrochemical experiments were performed on a BAS-100B electrochemical analyzer at room temperature. The electrochemical cell with a volume of 5 mL was equipped with a saturated calomel reference electrode, a platinum wire auxiliary electrode and a carbon paste working electrode. The real geometric area of the CPE was determined to be 1.9×10^{-3} cm^2 from the slope of the plot of the anodic peak current for 1.0 mmol dm^{-3} $K_3[Fe(CN)_6]$ in 0.1 mol dm^{-3} KCl *vs*. the square root of scan rate. All experimental solutions were deoxygenated by bubbling highly pure nitrogen for 15 min and maintained under nitrogen atmosphere during the course of the experiment. Amperometric experiments were carried out in a stable system operated at −900 mV upon successive additions of 5 μL 0.1 mol dm^{-3} $NaNO_2$.

Results and discussion

Electrochemical response of modified electrodes

Fig. 1 shows the cyclic voltammograms of CPE, Au–CPE, Hb–CPE and Hb–Au–CPE in a pH 5.5 NaAc–HAc buffer. Hb–Au–CPE exhibited a couple of stable and well-defined redox peaks at −(215 ± 1) and −(350 ± 2) mV at 150 mV s^{-1}. No peak was observed for either the CPE or the Au–CPE, at which the cyclic voltammograms displayed low background current. Thus, the response of Hb–Au–CPE was attributed to the redox of the electroactive center of the immobilized Hb. When Hb was mixed with carbon paste without the presence of gold colloid, the Hb–CPE also showed the response of Hb, indicative of the biocompatibility of the carbon paste. The response, however, was 2.5 times smaller than that of Hb–Au–CPE, and the peak-to-peak separation of 164 mV was larger than the 135 mV response of the Hb–Au–CPE. The formal potential ($E^{0\prime}$) of the heme $Fe^{III/II}$ couple of Hb in Hb–CPE, estimated as the midpoint of the anodic and cathodic peak potentials, was −307 mV at pH 5.5. For the Hb–Au–CPE, the value of $E^{0\prime}$ was −283 mV (−42 mV *vs*. NHE). The $E^{0\prime}$ of the heme $Fe^{III/II}$ couple of Hb in Hb–Au–CPE was close to the value of Hb in solution.[27] Thus, the colloidal gold played an important role in maintaining the biological integrity of Hb and facilitating the electron exchange between the Hb and carbon sensing sites. The positive shift in $E^{0\prime}$ of Hb in Hb–Au–CPE was due to the presence of negatively charged gold colloid nanoparticles, which stabilized the oxidized form of Hb.

The effect of the scan rate on the response of immobilized hemoglobin was shown in the insets in Fig. 1. The peak currents were proportional to the scan rate at scan rates of less than 80 mV s^{-1} (inset A in Fig. 1), indicating a surface-controlled electrode process. From the integration of the reduction peak of Hb–Au–CPE at 10, 20, 40 and 60 mV s^{-1}, an average total amount of hemoglobin (Q/nF) was calculated to be (9.90 ± 1.42) × 10^{-12} mol. When the scan rate was larger than 80 mV s^{-1} the peak currents were proportional to the square root of the scan rate, $v^{1/2}$ (in inset B of Fig. 1). At high scan rates the electrode reaction was not a surface-controlled electrode process.

Upon the decrease of scan rate the peak-to-peak separation decreased. At slow scan rates the peak-to-peak separation deviated from the theoretical value of 0 mV, which was probably ascribable to the immobilized hemoglobin molecules in various orientations and the slow electron transfer kinetics.

Influence of solution pH

Cyclic voltammograms of Hb in Hb–Au–CPE showed a strong dependence on solution pH. An increase of solution pH caused a negative shift in both cathodic and anodic peak potentials (Fig. 2). The plot of the formal potential *versus* pH (from 3.0 to 6.0) produced a line with a slope of −(55.4 ± 2.2) mV pH^{-1}, which is close to the expected value of −58.0 mV pH^{-1} for a single proton transfer coupled to a reversible single electron transfer process, indicative of one proton and one charge attending in the electron transfer process. The participation of a proton could be explained by the protonation of groups near the heme iron, in order to neutralize the excess charge that accumulated at the

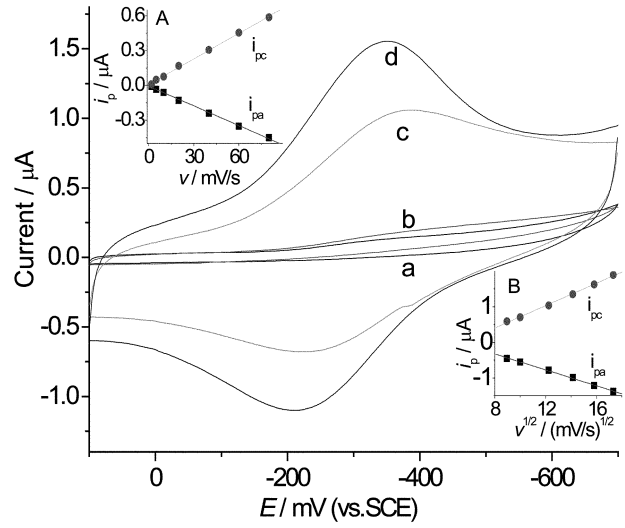

Fig. 1 Cyclic voltammograms of CPE (a) and Au–CPE (b), Hb–CPE (c) and Hb–Au–CPE (d) in 0.2 mol dm^{-3} pH 5.5 NaAc–HAc at 150 mV s^{-1}. Inset: plots of peak currents *vs*. v (A) and plots of peak currents *vs*. $v^{1/2}$ (B).

interface upon electrochemical reduction. Therefore, the electrode process can be expressed as follows:[28]

$$\mathrm{HbFe(III) + H^+ + e^- \rightleftharpoons HbHFe(II)} \quad (1)$$

The irreversible decrease in peak current at pH $\leq$ 3.5 reflected the influence of pH on protein denaturation due to the dissociation of the heme–globin complex between 4.3 and 2.5.[28]

Electrochemical response of NO_2^- at Hb–Au–CPE

Upon addition of a low concentration of $NaNO_2$ to pH 4.0 HAc–NaAc, both the cathodic and anodic peak currents of Hb in Hb–Au–CPE increased (Fig. 3). The increase in cathodic peak current of hemoglobin upon the addition of 20 μM $NaNO_2$ was 37 times higher than the reduction response of NO_2^- at −286 mV at Au–CPE. Thus, the peak was not a simple overlap of reduction peaks of HbFe(III) to HbHFe(II) and NO_2^- to NO. The increase value of the cathodic peak current (the total peak current subtracted from the peak current of Hb in the absence of NO_2^- at the same rate) was proportional to the square root of the scan rate, showing no difference from the EC behavior at a large value of k/v,[40] where k was the rate constant of the chemical reaction following the electrochemical reaction and v was the scan rate. The plot of the cathodic peak potentials *versus* the logarithm of the scan rates yielded a line with a slope of 35.4 mV near the theoretical value of 30 mV for an EC process, including a charge transfer followed by an irreversible chemical reaction. Thus, the increase of the cathodic current was attributed to the reduction reaction of HbFe(III) to HbHFe(II) (eqn. (1)) and NO_2^- to NO[41] (eqn. (2)) and the irreversible chemical reaction of NO bind rapidly to heme iron in Hb to form HbHFe(II)NO[38] (eqn. (3)), one interfacial charge transfer followed by a chemical reaction (EC process).

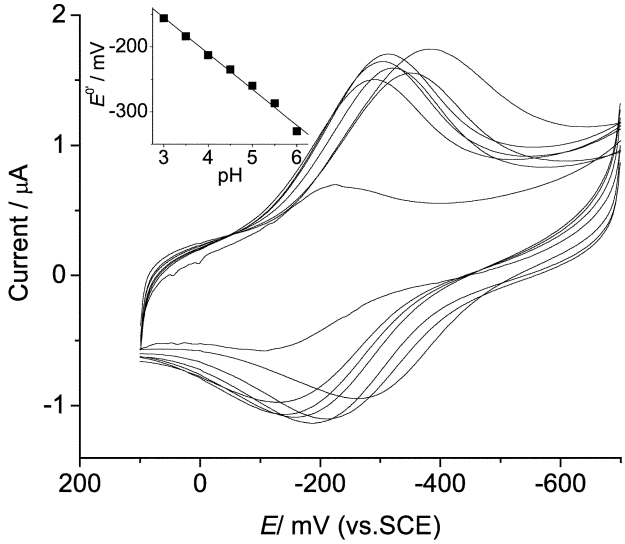

Fig. 2 Cyclic voltammograms of Hb–Au–CPE in pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 (from positive to negative peak potentials) 0.2 mol dm^{-3} NaAc–HAc at 150 mV s^{-1}. Inset: plot of $E^{0\prime}$ *vs.* pH

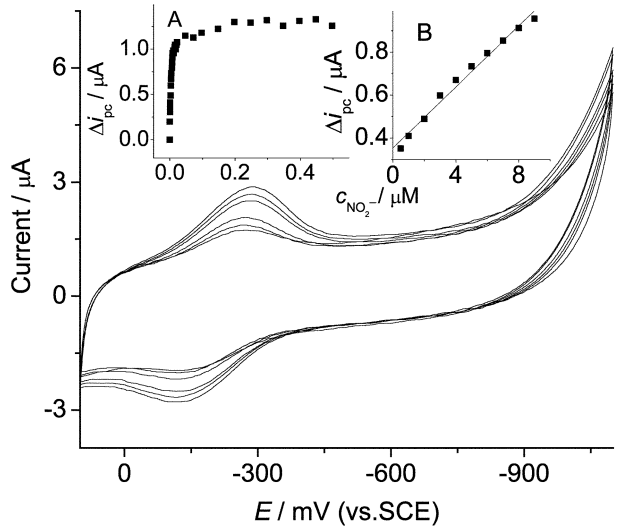

Fig. 3 Cyclic voltammograms of Hb–Au–CPE in pH 4.0 NaAc–HAc containing 0, 0.3, 1.0, 6.0, 10.0 and 14.0 μmol dm^{-3} $NaNO_2$ measured at 150 mV s^{-1}. Inset: plots of cathodic peak current increases *vs.* NO_2^- concentrations ranging from 0.1 to 500 μmol dm^{-3} (A) and 0.1 to 10 μmol dm^{-3} (B).

$$\mathrm{NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O} \quad (2)$$

$$\mathrm{HbHFe(II) + NO \rightarrow HbHFe(II)NO} \quad (3)$$

The presence of Hb in the Hb–Au–CPE facilitated the reduction reaction of NO_2^-. At high concentrations of nitrite the surface immobilized HbHFe(II)NO reached maximum, thus the cathodic peak current reached a saturation value (inset A in Fig. 3).

The increase of the oxidation peak current upon addition of NO_2^- was attributed to the oxidation reaction of HbHFe(II), and nitrosyl Hb, HbHFe(II)NO. The oxidation of HbHFe(II)NO with one electron transfer would firstly form the nitrosyliron(III) intermediate, HbHFe(III)NO, which was then followed by an autoreduction process in a weak acidic solution to form nitrite.[38,42] Thus, once again, the CV behavior would display an interfacial EC process including a charge transfer followed by an irreversible chemical reaction. The oxidation peak current was also proportional to the square root of the scan rate, and the plot of the anodic peak potentials *versus* the logarithm of scan rate gave a line with a slope of 36.2 mV. Thus, the electrode reaction can be described as follows:[38]

$$\mathrm{HbHFe(II)NO - e \rightleftharpoons HbHFe(III)NO} \quad (4)$$

$$\mathrm{HbHFe(III)NO \rightleftharpoons HbHFe(II)NO^+} \quad (5)$$

$$\mathrm{HbHFe(II)NO^+ + H_2O \rightarrow HbHFe(II) + NO_2^- + 2H^+} \quad (6)$$

The formed HbHFe(II) and free HbHFe(II) at low NO_2^- concentration were further oxidized at the same potential:

$$\mathrm{HbHFe(II) - e \rightleftharpoons HbFe(III) + H^+} \quad (7)$$

The combination of reaction (4)–(6) and reaction (7) resulted in the increase of the oxidation peak current.

Determination of NO_2^- at Hb–Au–CPE

At low $NaNO_2$ concentrations the plot of the increase in cathodic peak current due to the presence of the interfacial EC process *versus* $NaNO_2$ concentration displayed a linear range from 0.1 to 9.7 μmol dm^{-3} (inset B in Fig. 3). The linear regression equation was $y = 0.071x + 0.35$ μA, with a correlation coefficient of 0.993. From the slope of 0.071 μA μmol^{-1} dm^3 the detection limit was estimated to be 0.06 μmol dm^{-3} at 3σ. The sensitivity is half of that based on the electrocatalysis of the ruthenium polymer to NO_2^- oxidation,[12] corresponding to that based on the electrocatalytic reduction of nitrite,[15] yet higher than those based on the electrocatalysis of polyoxometalate[43] and the transition metal substituted Dawson polyanions[44] to NO_2^- reduction. The responses of four independently prepared electrodes for NO_2^- reduction in the linear range showed an acceptable reproducibility. The standard deviation of the response slopes was *ca.* 6.2%. The advantages of our biosensor, based on the new sensing mechanism, are its good stability, surface renewal (see below), commercial availability and low cost of the modifier, Hb.

Fig. 4 shows the cyclic voltammograms of Hb–Au–CPE upon successive additions of $NaNO_2$ to the bulk solution, pH 4.0 NaAc–HAc. At high $NaNO_2$ concentrations a new irreversible reduction wave occurred at about −0.72 V at 150 mV s^{-1}, which was attributed to the further reduction of NO and not observed when the concentration was lower than 0.1 mmol dm^{-3}. This response was similar to those reported previously at Hb–DNA film modified PGE.[32] The peak current of NO reduction increased upon successive addition of $NaNO_2$ within the calibration range from 0.1 to 4.0 mmol dm^{-3} (inset in Fig. 4). The linear relation between peak current and NO_2^- concentration was obtained in the range of 0.1 to 0.5 mmol dm^{-3} with a correlation coefficient of 0.998, corresponding to the biosensor based on the Hb–DNA films at pH 2.6.[32] Considering a lower disproportionation rate of NO_2^- to form NO and a smaller reduction peak current at pH 4.0 than those at pH 2.6, the biosensor based on the Hb–Au–CPE displayed a higher sensitivity for NO determination.

Fig. 5 shows a typical hydrodynamic current–time response of the Hb–Au–CPE at −900 mV upon successive additions of

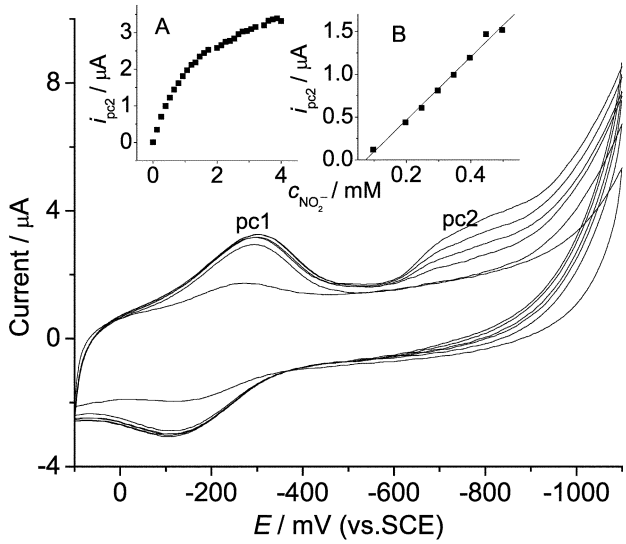

Fig. 4 Cyclic voltammograms of the Hb–Au–CPE in pH 4.0 NaAc–HAc containing 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mmol dm^{-3} $NaNO_2$ measured at 150 mV s^{-1}. Inset: plots of the reduction peak current *vs*. NO_2^- concentrations ranging from 0.1 to 4 mmol dm^{-3} (A) and 0.1 to 0.5 mmol dm^{-3} (B).

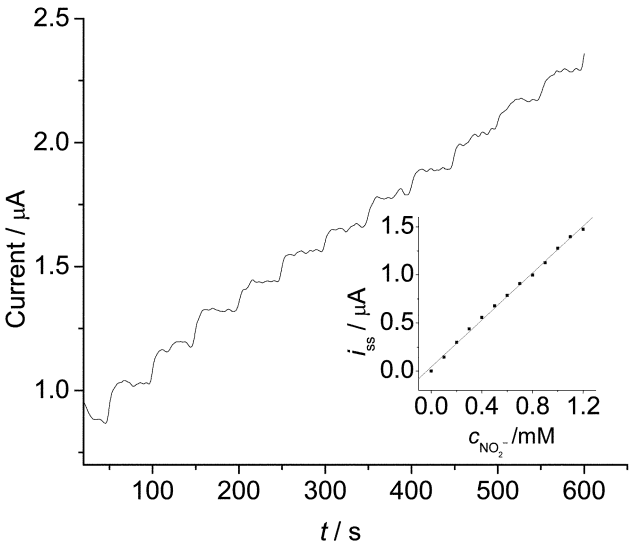

Fig. 5 Amperometric responses of the Hb–Au–CPE at −900 mV upon successive additions of 5 μL 0.1 mol dm^{-3} $NaNO_2$ to 5.0 mL pH 4.0 NaAc–HAc. Inset: linear relation between the amperometric response and NO_2^- concentration.

$NaNO_2$ to pH 4.0 NaAc–HAc buffer. Upon the addition, the sensor achieved 95% of its steady-state current within 10 s, displaying a fast amperometric response to NO reduction. The amperometric response showed a linear relation with nitrite concentration from 0.1 to 1.2 mmol dm^{-3} (inset in Fig. 5) with a correlation coefficient of 0.998 (n = 13). The sensitivity was better than the reported concentration limit of 5×10^{-4} mol dm^{-3} NO_2^-.[44]

Interferences

In order to assess the selectivity of the proposed biosensors, the influences of common foreign species were examined by adding these species into pH 4.0 NaAc–HAc buffer containing 1.0 μmol dm^{-3} NO_2^-. 10 μmol dm^{-3} dopamine, catechol, uric acid and epinephrine and 50 μmol dm^{-3} ascorbate, cystine and EDTA showed little interference to the NO_2^- determination. Metal ions, such as Cu(II), Cd(II), Pb(II), Mo(II), Cr(III), Mn(II), Fe(III) and Fe(II) at 50 μmol dm^{-3} did not interfere with the determination. The interference of co-existing metal ions with higher concentrations could be eliminated by adding EDTA in the sample.

Stability and renewal of nitrite biosensor

After the Hb–Au–CPE was stored at 4 °C for two weeks, no obvious decrease in the response to NO_2^- was observed. After a 30 day storage period, the sensor retained 91% of its initial current response. Thus colloidal gold mixed in carbon paste was very efficient for retaining the activity of Hb and preventing it from leaking out of the sensor. After the electrode was used in a pH 4.0 solution for 6 h, the response of the sensor decreased irreversibly due to the dissociation of immobilized Hb.[28] The surface with denatured Hb could be renewed by gently rubbing it on a fine piece of paper. The current response of the renewed surface was examined at a NO_2^- concentration of 20 μmol dm^{-3}. The relative standard deviation was 5.2% for six successive renewals. Thus, the method was rapid, easy and, more importantly, reproducible for removing the surface of the denatured Hb film.

Determination of nitrite in the water of Taihu Lake

The determination of nitrite in water samples from Taihu Lake was carried out with this biosensor. The nitrite levels in two samples obtained at different sites were determined to be 2.13 ± 0.04 μmol dm^{-3} (0.098 ppm) and 2.04 ± 0.16 μmol dm^{-3} (0.093 ppm), respectively. These values were very close to the results of 0.1 ppm and 0.09 ppm measured with spectroscopy at 540 nm by adding *p*-aminobenzsulfamide to samples to form a diazonium salt and then reacting with *N*,*N*′-(1-naphthyl)-ethylenediamine to form a red compound. The recoveries for the analyses of 0.5–5.0 μmol dm^{-3} nitrite were between 97–104%. The results were satisfactory. Obviously, the method presented in this paper is simpler and more rapid than the latter.

Conclusions

Colloidal gold nanoparticles facilitate the electron exchange between the hemoglobin immobilized in gold colloid nanoparticle modified carbon paste and carbon sensing sites and amplify the electrochemical information. Upon addition of NO_2^- to the supporting electrolyte, the increases in both the cathodic and anodic peak currents of the immobilized hemoglobin is observed, which provides a sensitive method for NO_2^- detection. The improvement in sensitivity and detection limit

attributes to the observed interfacial EC process. Thus, the interfacial EC process would possess the same importance and scientific significance as the electrocatalytic processes in analytical application. The biosensor shows a good stability and can be renewed quickly and reproducibly by a simple polish step.

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