J Solid State Electrochem (2010) 14:1681–1688
DOI 10.1007/s10008-010-1010-3

ORIGINAL PAPER

The immobilization of Cytochrome *c* on MWNT–PAMAM–Chit nanocomposite incorporated with DNA biocomposite film modified glassy carbon electrode for the determination of nitrite

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Received: 25 September 2009 / Revised: 14 January 2010 / Accepted: 17 January 2010 / Published online: 17 February 2010
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Abstract A novel and sensitive biosensor was developed for the determination of nitrite. Firstly, multi-walled carbon nanotubes–poly(amidoamine)–chitosan (MWNT–PAMAM–Chit) nanocomposite along with the incorporation of DNA was used to modify the glassy carbon electrode. Then the immobilization of Cyt *c* was accomplished using electrochemical deposition method by consecutive cyclic voltammetry (CV) scanning in a neutral Cyt *c* solution. CV behaviors of the modified electrodes showed that the MWNT–PAMAM–Chit nanocomposite is a good platform for the immobilization of DNA and Cyt *c* in order, at the same time, an excellent promoter for the electron transfer between Cyt *c* and the electrode. At high potential, the immobilized Cyt *c* could be further oxidized into highly reactive Cyt *c* π-cation by two-step electrochemical oxidation, which could oxidize NO_2^- into NO_3^- in the solution. Therefore, a nitrite biosensor based on the biocatalytic oxidation of the immobilized Cyt *c* was fabricated, which showed a fast response to nitrite (less than 5 s). The linear range of 0.2–80 μM and a detection limit of 0.03 μM was obtained. Finally, the application in food analysis using sausage as testing samples was also investigated.

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Keywords MWNT–PAMAM–Chit nanocomposite · Cytochrome *c* · DNA · Nitrite biosensor · High reactive Cyt *c* π-cation

Introduction

It is important to study the electrochemical behavior of Cyt *c* because of its critical role in the biological respiratory chain. A lot of compounds have been found to promote the electron transfer between Cyt *c* and electrode because it is difficult for Cyt *c* to undergo a facile redox process at conventional electrodes [1, 2]. Recently, more and more attention has been focused on the fabrication of Cyt *c* modified electrode, and the application of those Cyt *c* modified electrodes was no longer limited to the detection of hydrogen peroxide (H_2O_2). A mass of studies have reported its application in the determination of nitric oxide [3], nitrite [4, 5], superoxide radical anion [6], and so on.

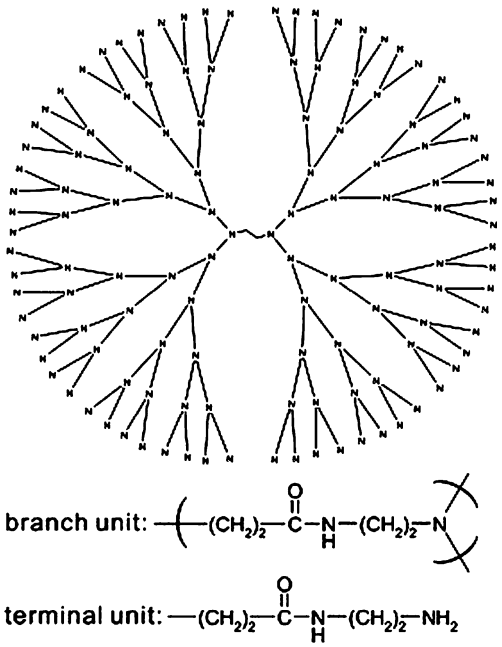
Nitrite, which is usually used as preservative, exists widely in water, food, and physiological systems. But it is found that the nitrite anion can easily interact with amines to form carcinogenic nitrosoamines [7]. So it is necessary to develop effective methods for the determination of nitrite for environmental reason and human health. Nowadays, many methods such as spectroscopic analysis [8], capillary electrophoresis [9], polarographic analysis [10], chromatography [11], and electrochemical methods [12–14] have been developed for the nitrite determination. Among them, the electrochemical methods, especially electrochemical biosensors, possess a lot of advantages such as rapid response, high sensitivity, low-cost, time-saving, and simple use. Therefore, much attention has been focused on the development of high-performance nitrite electrochemical biosensors. Most

nitrite biosensors were attributed to the catalysis of proteins for the reductive reaction of nitrite which is complicated and the products are complex [15–17]. However, a new kind of nitrite biosensor was reported lately which was based on the Cyt *c* modified electrode for electrochemical oxidation of nitrite [4]. The immobilized Cyt *c* could be further oxidized at high potential, and the resulted high reactive Cyt *c* π-cation could oxidize NO_2^- into NO_3^- in the solution.

Poly(amidoamine) (PAMAM) dendrimers, in which the amino groups with high density are easily functionalized by other substances, exhibit good chemical properties, and spherical morphologies [5]. The fourth (G4; Scheme 1) or higher generation PAMAM dendrimers are approximately spherical molecules. In our previous reports [18, 19], the G4 PAMAM dendrimers was proved to be good for DNA immobilization because of its abundant amino groups, good biocompatibility, and quite open spherical 3D structures at high generation [20]. In view of that, DNA-modified electrode has been proven to be seized of the ability to immobilize Cyt *c* [21, 22], and the capacity to immobilize DNA will direct influence the accuracy and sensitivity of the obtained Cyt-*c*-modified electrode. PAMAM dendrimers could act as a good platform for the DNA immobilization and further for the electrochemical deposition of Cyt *c* in the solution.

Carbon nanotubes (CNTs) are hexagonal networks of carbon atoms with a large delocalized π-π conjugate electron structure forming seamless cylinders [23]. CNTs possess many unique properties such as nanometer size, catalytic activity, excellent chemical, and physical stability, showing great promise of developing and application nanocomposites in various fields especially electroanalysis [24]. Chitosan (Chit), the main component of the shell of shrimps, crabs, and insects, has not only hydrophilic and hydrophobic groups but also complex groups such as $–NH_2$ and –OH [25]. It has been widely used as the modifying reagent to prepare chemically modified electrode.

In this study, G4 PAMAM dendrimers and multi-walled carbon nanotubes (MWNT) were dispersed in 0.2 wt.% Chit solution to give MWNT–PAMAM–Chit nanocomposite. The resulting nanocomposite along with the incorporation of DNA and Cyt *c* was firstly used to modify the glassy carbon electrode (GCE). The redox behavior of Cyt *c*, the electrochemical response of the modified electrode to nitrite and its application in food analysis were detailed herein.



Scheme 1 Schematic drawing of the molecular structure of the G4 PAMAM dendrimer

Experimental

Reagents

Chitosan was obtained from Aldrich and a 0.2 wt.% chitosan solution was prepared by dissolving chitosan in 1 wt.% acetic acid solution. Horse heart Cytochrome c (Cty c, MW 12384) and DNA (Calf Thymus) were purchased from Sigma and used without further purification. One millimolar Cyt *c* stock solution was prepared by 0.1 M pH 7.0 phosphate buffer solution (PBS), which was prepared by mixing the stock solutions of Na_2HPO_4 and NaH_2PO_4, and stored at a temperature of 4 °C. DNA stock solutions (100 ppm) were prepared with TE solution (10 mM Tris–HCl, 1 mM EDTA, pH 8.00) and kept frozen. G4 PAMAM (Scheme 1) [26] dendrimers were synthesized according to the reported procedure [27, 28]. A methanol solution of 20% G4 PAMAM dendrimers was purified by dialyzing. MWNT were purchased from Nachen S&T Ltd (Beijing, China) and treated according to reported procedure [29]. *N*-hydroxysulfosuccinimide (NHS) and 1-[3-(dimethylamino) propyl]−3-ethylcarbodiimide hydrochloride (EDC) were obtained from Fluka. All other materials were of at least analytical grade and used without further purification. Doubly distilled and deionized water was used throughout this work.

Preparation of the modified electrodes

Before modification, the bare GCE was polished to a mirror-like surface with 0.05 μm Al_2O_3 slurry, then rinsed with water, ultrasonicated in 1:1 (*v*/*v*) HNO_3, ethanol, and doubly distilled water, respectively. It was dried under the stream of high purity nitrogen for further use. MWNT, 0.1 mg, and 50 μL solution of 20% PAMAM dendrimers in methanol were homogeneously dispersed in 1 ml 0.2 wt.% chitosan

solution at room temperature with the aid of ultrasonic agitation. Then 7.5 μL of the resulting mixture was firstly dropped onto the cleaned GCE surface to obtain MWNT–PAMAM–Chit/GCE. Secondly, 5 μL of the DNA stock solutions (100 ppm) was dropped onto the MWNT–PAMAM–Chit/GCE surface, which was treated with 0.4 mM EDC–0.1 mM NHS, and reacted for 2 h to give DNA/MWNT–PAMAM–Chit/GCE. Finally, to immobilize Cyt *c* onto the above electrode, the electrochemical deposition of Cyt *c* was performed in 0.1 M pH 7.0 PBS solution containing 0.1 mM Cyt *c* by consecutive CV over a suitable potential region of −0.1 to 0.6 V. The electrode referred in the text as Cyt *c*/DNA/MWNT–PAMAM–Chit/GCE was obtained and preserved in a refrigerator at 4 °C after being washed with 0.1 M pH 7.0 PBS solution.

Electrochemical measurements

All the electrochemical measurements were performed by using a CHI 832a electrochemical workstation (Chenhua Co., Shanghai, China) at room temperature. A conventional three-electrode system was used in the measurements with a saturated calomel electrode as the reference, a Pt wire as the auxiliary electrode, and a bare or a modified electrode as the working electrode. CV was conducted in 5 mM $K_3Fe(CN)_6$ solution and 0.1 M pH 7.0 PBS containing $NaNO_2$ with different concentrations.

Determination of nitrite in food samples

The sausage, used as the testing samples in food analysis, were bought in a local supermarket and pre-treated as the reported procedures [30].

Results and discussion

Cyclic voltammetric behaviors of the modified electrodes in $K_3Fe(CN)_6$ solution

Cyclic voltammetric method was carried out in 5 mM $K_3Fe(CN)_6$ solution to investigate the changes on the electrode surface aroused from every surface modification step as shown in Fig. 1. The anodic and cathodic peak currents of the four electrodes were also shown in the insert. The peaks of the bare GCE (Fig. 1a) at 0.19 and 0.27 V were attributed to the oxidation and reduction of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$, respectively. Compared with bare GCE, both oxidative and reductive peaks of PAMAM–MWNT–Chit nanocomposite modified electrode enlarged dramatically (Fig. 1b). This phenomenon may be noted that: (1) the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ could be strongly attracted by the amino groups of MWNT–PAMAM–Chit nanocomposite on the electrode surface and (2) the electrochemical reactions could be greatly catalyzed by MWNT contained in PAMAM–MWNT–Chit nanocomposite. However, the peak currents of DNA/MWNT–PAMAM–Chit/GCE reduced a lot as shown in Fig. 1c, which indicated that DNA had been successfully immobilized on the electrode surface, because the negatively charged phosphate groups of DNA will block the electrochemical reactions on the electrode surface. As presented in Fig. 1d, the peak currents increased obviously when the electrode was finally modified with Cyt *c*. Because the positively charged lysine residue of Cyt *c* could provide a better platform for the electrochemical reactions of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ than that of DNA, which is in close accord with the previous report [5]. It appeared that Cyt *c* was immobilized on the DNA/MWNT–PAMAM–Chit/GCE.

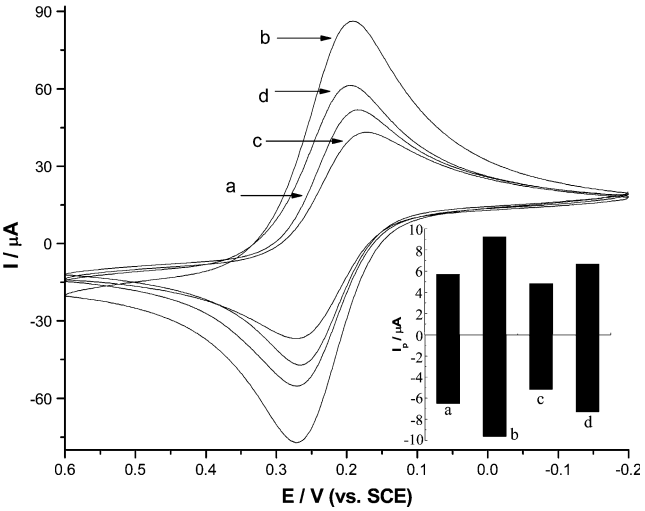


Fig. 1 CVs of the GCE (**a**), MWNT–PAMAM–Chit/GCE (**b**), DNA/MWNT–PAMAM–Chit/GCE (**c**), and Cyt *c*/DNA/MWNT–PAMAM–Chit/GCE (**d**) in 5 mM $K_3Fe(CN)_6$ solution at 0.1 V s^{-1}; *insert* the anodic and cathodic peak currents (i_p) of the four curves

Electrochemical behavior of Cyt *c* on modified electrodes

Figure 2 showed the CVs of Cyt *c* on different modified electrodes in 0.1 M pH 7.0 PBS at 0.05 V s^{-1}. Cyt *c*/DNA/GCE (Fig. 2a) did not show any peaks since it is difficult for Cyt *c* to undergo a facile redox process at DNA-modified GCE. Compared with Cyt *c*/DNA/Chit/GCE (Fig. 2b), a pair of clear redox peaks of Cyt *c*/DNA/PAMAM–Chit/GCE appeared in Fig. 2c, which could be interpreted as the abundant amino groups of PAMAM dendrimers, provided more binding sites for the immobilization of DNA and finally the fixation of Cyt *c*. It is worth pointing out that PAMAM–MWNT–Chit nanocomposite

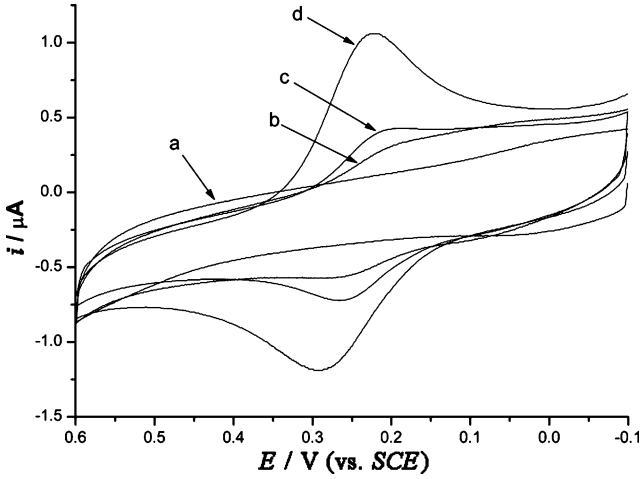


Fig. 2 CVs of Cyt *c*/DNA/GCE (**a**), Cyt *c*/DNA/Chit/GCE (**b**), Cyt *c*/DNA/PAMAM–Chit/GCE (**c**), and Cyt *c*/DNA/MWNT–PAMAM–Chit/GCE (**d**) in 0.1 M pH 7.0 PBS at 0.05 V s^{-1}

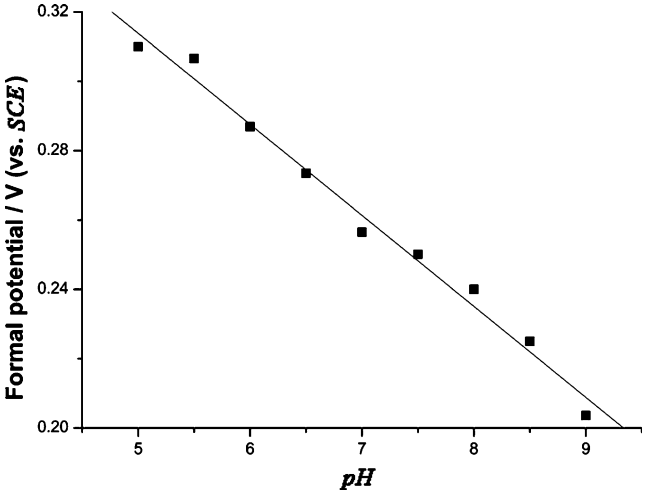


Fig. 3 Plots of the formal potential of Cytc/DNA/MWNT–PAMAM–Chit/GCE vs. the solution pH value

was used for the electrode modification (Fig. 2d), the peak currents were about three times than that of Cyt *c*/DNA/PAMAM–Chit/GCE, indicating that PAMAM–MWNT–Chit nanocomposite could act as both a perfect platform for the immobilization of DNA and an excellent promoter for the electron transfer between Cyt *c* and the electrode.

Effect of pH value

The effect of the solution pH value on the formal potential of Cyt *c*/DNA/MWNT–PAMAM−Chit/GCE was investigated by cyclic voltammetry method. Nearly reversible voltammograms with stable and well-defined redox peaks were obtained in the pH range of 5.0∼9.0, and the increase of solution pH resulted in negative shift of the formal potential as shown in Fig. 3. A linear relationship was obtained between the formal potential and pH with the slope of 28.5 mV pH^{-1}, suggesting that the electrochemistry reaction of Cyt *c* involved a proton process [4]. The slope is much smaller than the theoretical value (59 mV pH^{-1}) for the electrons transfer accompanied with an equal number of protons in electrode reaction, which could be attributed to the influence of the protonation states of trans ligands to the heme iron and amino acids around Cyt *c*, or the protonation of the water molecule coordinated to the central iron [5, 31].

Investigation of DNA coverage amount

The full coverage of DNA on the modified electrode surface was investigated in order to get the optimize conditions for the electrochemical deposition of Cyt *c*. The study was performed by immobilizing different concentrations of DNA on the surface of MWNT–PAMAM–Chit/GCE. The obtained DNA modified electrodes were used for the electrochemical deposition of Cyt *c* in the same way. The peak currents of the immobilized Cyt *c* on those modified electrodes in the bulk PBS solution was recorded as shown in Fig. 4. Both the anodic and cathodic peak currents first increased with the increasing DNA concentration, and then decreased remarkably beyond 100 ppm, at which the full coverage of DNA was achieved. As a result of higher quantities of DNA, space steric hindrance would prevent DNA adsorption on the surface of modified electrode at higher concentrations [32, 33], and finally blocked the electrochemical deposition of Cyt *c*. Therefore,

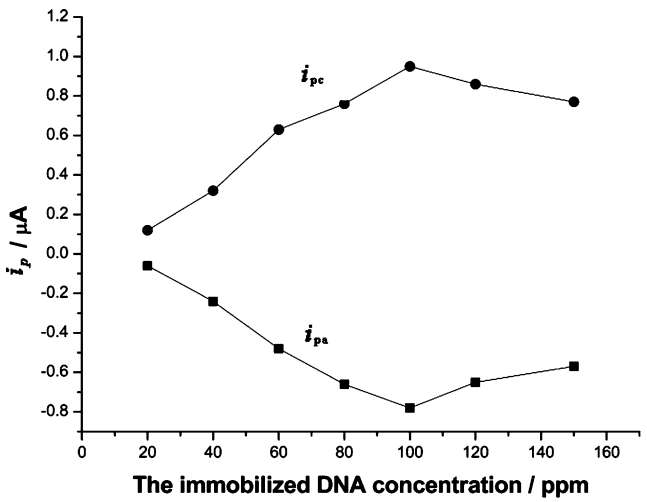


Fig. 4 Influence of different DNA coverage amount on the anodic and cathodic peak currents of the immobilized Cyt *c*

this DNA concentration of 100 ppm was suitable for Cyt *c* immobilization onto the DNA/MWNT–PAMAM–Chit/GCE.

Effect of scan rate

The effect of scan rates on the voltammetric behavior of Cyt *c* was investigated in order to further investigate the characteristics of the immobilized Cyt *c* on the modified GCE as shown in Fig. 5. The cathodic and anodic peak currents linearly increased with the scan rate (υ) from 0.05 to 0.5 V s^{-1} (shown in insert a of Fig. 5). In other words, the current function (i_p/υ) had a constant value at different scan rates, indicating that the electrode reaction of Cyt *c* immobilized on the modified electrode was mainly controlled by the surface-controlled processes.

In addition, the oxidation peak shifted to more positive potentials, while the reduction peak shifted to more negative potentials with increasing of scan rate. By plotting E_p vs. logυ, two linear relationships were observed when $\upsilon >$ 0.25 V s^{-1} as shown in inset b of Fig 5. On the basis of Laviron's method [34]: a graph of $E_p = f(\log\upsilon)$ yields two straight lines with a slope equal to $-2.3RT/\alpha nF$ for the cathodic peak, and $2.3RT/(1-\alpha)nF$ for the anodic peak, the charge transfer coefficient, α, was estimated to be 0.59. The average electron transfer rate constant, k_s=1.5 s^{-1}, can be obtained according to Eq. (1). This k_s value is slightly lower than that of our previous study (2.4 s^{-1}) [5], but it was higher than that of the first nitrite biosensor based on Cyt *c* (1.39 s^{-1}) [4] and other Cyt *c* modified electrodes such as Cyt *c*/Nb_2O_5 electrode (0.28 s^{-1}) [35] and Cyt *c*/NaY/GCE (0.78±0.04 s^{-1}) [36]. It denoted that the biocomposite film containing MWNT–PAMAM–Chit nanocomposite along with the incorporation of DNA is a good platform for the immobilization of Cyt *c*, at the same time, an excellent promoter for the electron transfer between Cyt *c* and the electrode.

$$\log k_s = \alpha\log(1-\alpha) + (1-\alpha)\log\alpha - \log(RT/nF\upsilon) - \alpha(1-\alpha)nF\Delta E_p/2.3RT \quad (1)$$

According to $Q=nFA\Gamma$, where Q is the charge involved in the reaction, n is the number of electron transferred, F is the Faraday's constant, and A is the electrode area (herein, the geometric area of GCE is used). The surface coverage (Γ) of Cyt *c* immobilized on the surface of the modified electrode could be calculated to be 8×10^{-10} mol cm^{-2}, which was much higher than the theoretical monolayer coverage of 1.4×10^{-12} mol cm^{-2} [37]. Because G4 PAMAM dendrimers are approximately spherical molecules with higher amino group densities, a three dimensional space will be established for the connection between the positive amino groups of PAMAM dendrimers and negative phosphate groups of DNA. As a result, the real area for the immobilization of Cyt *c* through electrostatic adherence is much higher than that of the electrode geometric area for the estimation of surface coverage.

Biocatalytic oxidation of Cyt *c*/DNA/MWNT–PAMAM–Chit/GCE to nitrite

Cyt *c* modified electrodes have been proven to be good biosensors for the determination of nitrite based on the biocatalytic oxidative property of Cyt *c* to nitrite: ferrous Cyt *c* can be oxidized to ferric form at low potential and the ferric Cyt *c* can be further oxidized at higher potential into [Fe^{4+}-Cyt *c*]·corresponding to step (I) and (II) of Eq. (2), respectively. [Fe^{4+}-Cyt *c*]·is a high reactive Cyt *c* π-cation

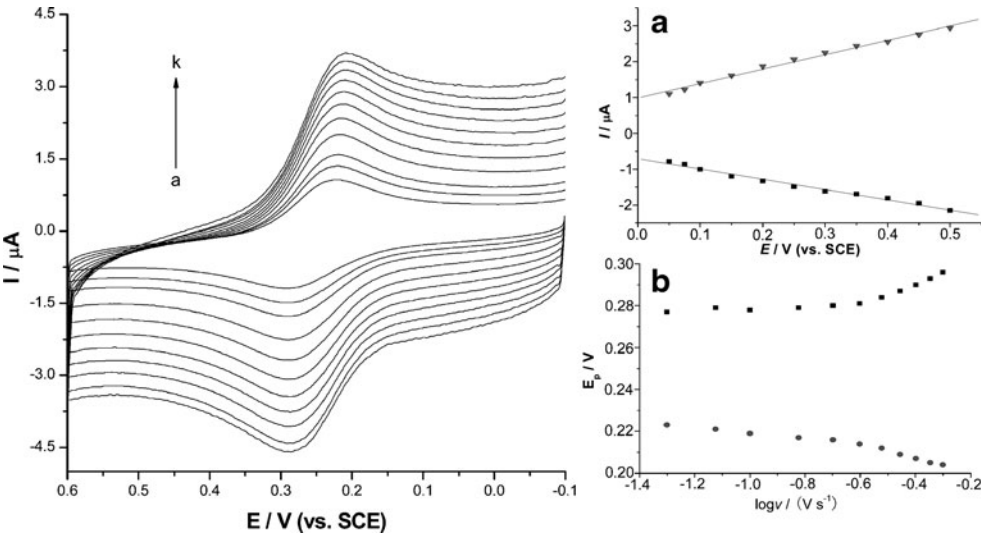
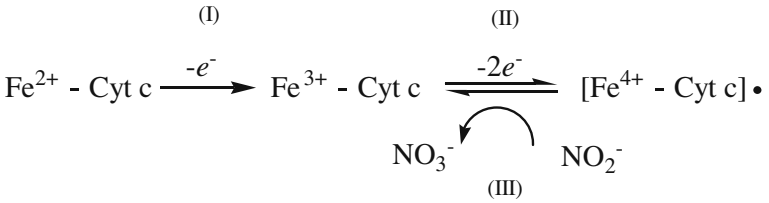


Fig. 5 CVs of Cyt *c*/DNA/MWNT–PAMAM–Chit/GCE at different scan rates in 0.1 M pH 7.0 PBS. Scan rates from (*a*) to (*k*) are 0.05, 0.075, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, and 0.5 V s^{-1}, respectively. Insert of **a** plots of anodic and cathodic peak currents (i_p) vs. the scan rate. Insert of **b** variation of anodic and cathodic peak potentials (E_p) vs. the logarithm of scan rate

possessed the ability to oxidize NO_2^- into NO_3^- in the solution (step (III) of Eq. (2)) [4, 5]. In this work, the oxidation of Cyt *c* and its biocatalytic oxidative property to nitrite was studied.



(2)

Figure 6a showed the result obtained from investigation of the CVs of Cyt *c*/DNA/MWNT–PAMAM–Chit/GCE in 0.1 M pH 7.0 PBS. There were two peaks appeared around 0.8 V (peak I) and 1.0 V (Peak II), respectively. Peak I was attributed to the oxidation of guanine of DNA, and peak II may be noted as an overlapping oxidation including the further oxidation of Cyt *c* and the oxidation of adenine of DNA according to the previous reports [4, 5, 38]. The further oxidation of Cyt *c* was confirmed by the investigation of the biocatalytic oxidative property of Cyt *c*/DNA/MWNT–PAMAM–Chit/GCE to nitrite. As shown in Fig. 6b(a), the CVs of Cyt *c*/DNA/MWNT–PAMAM–Chit/GCE in 0.1 M pH 7.0 PBS containing 2 μM $NaNO_2$ showed an obviously oxidation peak (peak III) at 0.95 V. What is more, this peak current increased obviously when the concentration of $NaNO_2$ reached 0.05 mM (curve b), which was in good agreement with our previous report [5]. Therefore, further oxidation of Cyt *c* occurred due to the oxidation of the high reactive Cyt *c* π-cation to $NaNO_2$ in solution. It is also important that the peak current was about ten times than that of the bare GCE (Fig. 6b(c)) indicating that the modified electrode catalyzed the oxidation of $NaNO_2$ greatly.

The determination of nitrite was carried out by the amperometric method on Cyt *c*/GA/MWNT–PAMAM–Chit/GCE at 0.95 V upon successive addition of different concentrations of $NaNO_2$ as shown in Fig. 7a. With the addition of $NaNO_2$ at regular speed into the gentle stirring PBS solution, a steady state current could be attained within less than 5 s, indicating a very rapid response to the change of $NaNO_2$ concentration. The linear response range of the biosensor to $NaNO_2$ concentration is from 0.2 to 80 μM. The detection limit of 0.03 μM obtained at a signal-to-noise ratio of 3 is much lower than that of other nitrite sensors and biosensors such as MnO_2/QPOE composite electrodes (0.36 μM) [39], Hb/HS-CdS-modified GCE (0.08 μM) [12] and Mb-ZnO-modified GE (4 μM) [40], indicating that this proposed method could potentially be used for monitoring

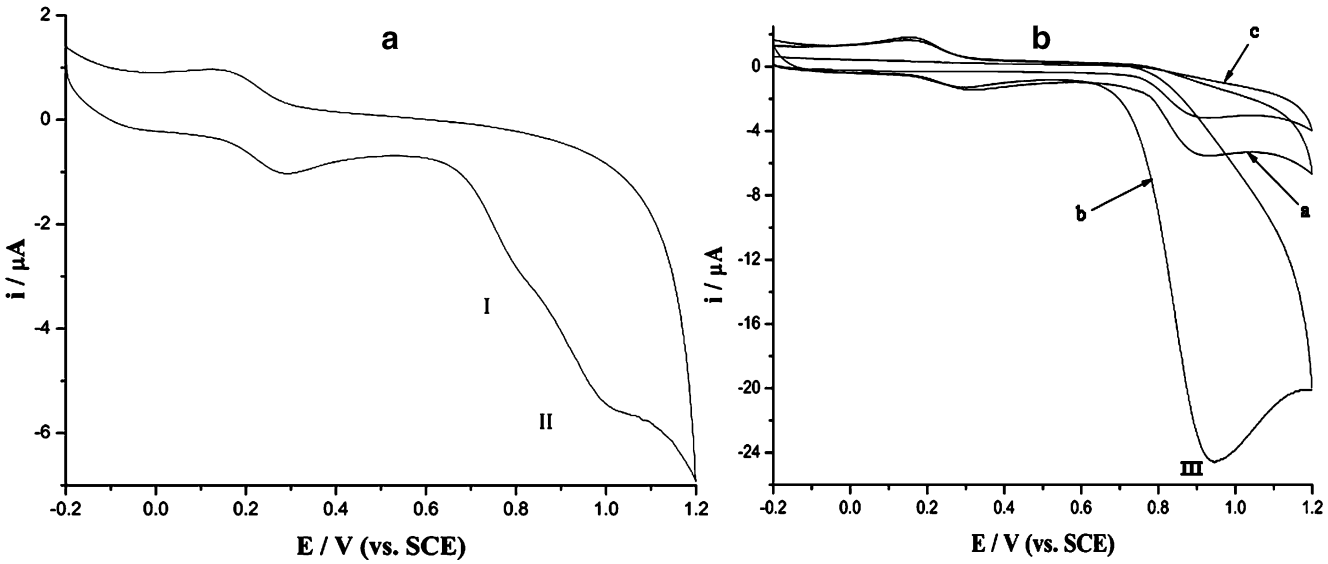


Fig. 6 **a** CVs of Cyt *c*/DNA/MWNT–PAMAM–Chit/GCE in 0.1 M pH 7.0 PBS at 0.05 V s^{-1}; **b** CVs of Cyt *c*/DNA/MWNT–PAMAM–Chit/GCE in 0.1 M pH 7.0 PBS containing 0.05 mM (*a*) and 2 μM $NaNO_2$ at 0.05 V s^{-1}, CVs of bare GCE (*c*) in 0.1 M pH 7.0 PBS containing 0.05 mM $NaNO_2$ at 0.05 V s^{-1}

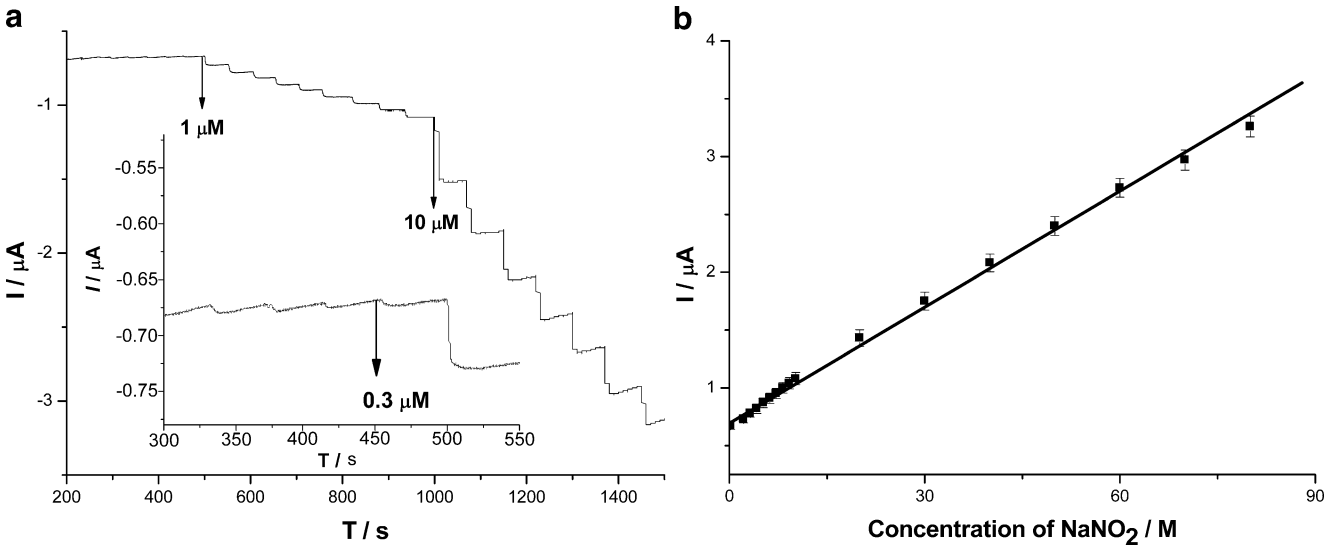


Fig. 7 **a** Typical current–time curve of the Cyt *c*/DNA/MWNT–PAMAM–Chit/GCE upon the successive addition of 0.3, 1, and 10 μM $NaNO_2$ into gently stirred 0.1 M pH 7.0 PBS. Applied potential, 0.95 V; **b** linear relation between the amperometric response and $NaNO_2$ concentration

of the concentration of $NaNO_2$ sensitively. The lower detection limit could be ascribed to the high loading of DNA for the immobilization of Cyt *c* and the rapid electron transfer between them.

To investigate the stability and reproducibility of the biosensor, the bare GCE was modified in the same way for seven times. The peak currents of the immobilized Cyt *c* in 0.1 M pH 7.0 PBS and for the determination of $NaNO_2$ at 40 μM showed acceptable reproducibility with RSD of 4% and 5%, respectively. After stored in 0.1 M pH 7.0 PBS at 4 °C for 1 month, the biosensor still retained about 90% of its initial sensitivity for the determination of $NaNO_2$.

The selectivity of the biosensor was studied by investigating the effects of some cations, anions, and small biomolecules on the current responses of 10 μM $NaNO_2$. The results showed that 50-folds of K^+, Na^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, F^-, Cl^-, NO_3^-,SO_4^{2-},PO_4^{3-}, and H_2O_2 and 40-folds of dopamine, L-tryptophane, uric acid, and glucose had almost no influences on the determination of nitrite, indicating that the biosensor had a good selectivity to nitrite.

Determination of nitrite in food samples

In order to examine the possible use of the proposed electrode in a practical application, experiments were studied in three sausage samples for determination of nitrite using the standard addition method. The results were listed in Table 1. In all cases, the RSD for each sample was less than 5%. The recoveries for the method were investigated and the values were obtained to be 101.5%, 100.6%, and 98.9%, respectively. The experimental data indicated that this proposed electrode could be successfully applied for the detection of nitrite in real samples.

Conclusions

A biocomposite film containing MWNT–PAMAM–Chit nanocomposite along with the incorporation of DNA has been used to modify glassy carbon electrode. Then, Cyt *c* was successfully immobilized on the modified electrode to fabricate a novel biosensor. Cyclic voltammetric method was also used to investigate the changes on the electrode surface aroused from every modification step and the influences of chitosan, PAMAM, and MWNT on the activity of the immobilized Cyt *c*. At high potential, the further oxidation of Cyt *c* occurred and the obtained Cyt *c* π-cation could oxidize NO_2^- into NO_3^- which made a sensitive response to the $NaNO_2$ concentration change. The biosensor showed a good linear range, a low detection limit, good reproducibil-

Table 1 Determination of nitrite in three sausage samples

Sample	Content (μM)	Added (μM)	Found (μM)	RSD (%)	Recovery (%)
1	1.50	2.50	4.06	4.24	101.5
2	2.20	2.50	4.73	2.12	100.6
3	3.10	2.50	5.54	4.24	98.9

ity, and stability. It has been successfully demonstrated for detection of nitrite in food material.

Acknowledgments This work was supported by the National Natural Science Foundation of China (No.20775044) and the Natural Science Foundation of Shandong province, China (Y2006B20).

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