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PAPER

A sensitive choline biosensor using Fe_3O_4 magnetic nanoparticles as peroxidase mimics

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A sensitive choline biosensor using Fe_3O_4 magnetic nanoparticles and a choline oxidase modified gold electrode was developed. Fe_3O_4 magnetic nanoparticles as peroxidase mimics used in the choline biosensor can not only improve the sensitivity of the response signal, but also possess the favorable properties of stability, magnetic separation and easy preparation, *etc.* When using the reduction currents of square wave voltammetry as the detection signals, the interferences of ascorbic acid and uric acid to the choline biosensor can be reduced effectively. The reduction currents of the square wave voltammetry were increased with the logarithm values of the choline chloride concentration (from 10^{-9} to 10^{-2} M), the detection limit was estimated to be 0.1 nM (S/N = 3). This choline biosensor also exhibited favorable selectivity and stability in the determination of choline chloride.

1. Introduction

There has been an intense interest in the use of nanoparticles for a wide range of biomedical and technological applications during the past two decades. Among the known nanomaterials, magnetic nanoparticles (MNPs), such as Fe_3O_4 MNPs, are of particular interest in magnetic resonance imaging, drug delivery, biological separation, and even in biological catalysis.[1–5] As MNPs themselves are usually thought to be chemically and biologically inert, they are typically coated with enzymes or metal catalysts to form dual-functional nanomaterials with both catalytic and magnetic separation properties.[6–9] For example, horseradish peroxidase (HRP)-entrapped magnetite-containing spherical silica nanoparticles have been used in biological catalytic and magnetic separation immunoassay systems.[4] However, recently, Yan's group has found that Fe_3O_4 MNPs themselves in fact possess an intrinsic enzyme mimetic activity similar to that found in natural peroxidases.[10] Most importantly, Fe_3O_4 MNPs enzyme mimics have some advantages over traditional natural enzymes. (1) Fe_3O_4 enzyme mimics are more stable than natural peroxidases, such as HRP, which is susceptible to the reaction conditions (such as temperature, pH, *etc.*), and even can be digested by proteases. (2) Fe_3O_4 enzyme mimics have the properties of magnetic separation and enrichment, which can facilitate biological applications. (3) The preparation and purification of natural enzymes are usually time-consuming and expensive, while the preparation of Fe_3O_4 MNPs is simple and economical.[11] Therefore, the robustness of Fe_3O_4 MNPs peroxidase mimics makes them suitable for a broad range of applications in the biological and medical fields.[12,13]

Choline is not only a precursor for biosynthesis of the neurotransmitter acetylcholine but also an important source of labile methyl groups. In addition, choline-containing phospholipids are ubiquitous components of various biological membranes.[14] Choline deficiency can affect memory and easily cause liver dysfunction or cancer, and moreover abnormal metabolism of choline has been implicated in a number of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases.[15,16] Therefore, Academy of Pediatrics, Food and Nutrition Board in some countries have established some standards for infants and adults about the daily intakes of choline.[17] Therefore, the determination of choline is crucial importance. Some enzyme-based biosensors, including the single enzyme (choline oxidase) or bi-enzyme (choline oxidase/horseradish peroxidase) sensors have been fabricated for the detection of choline.[18–23] Choline is catalyzed by choline oxidase in the presence of oxygen, and produces hydrogen peroxide. Therefore, we can detect choline through measuring the quantity of generated hydrogen peroxide.

$$\text{Choline} + H_2O + 2O_2 \xrightarrow{\text{Choline oxidase}} \text{Betaine aldehyde} + 2H_2O_2$$

In this paper, a sensitive choline biosensor was fabricated based on a Fe_3O_4 MNPs and choline oxidase modified gold electrode. Amino functionalized Fe_3O_4 MNPs were assembled on the cysteamine modified gold electrode surface through glutaraldehyde coupling. Then choline oxidase was immobilized on the modified electrode also through glutaraldehyde linking. The assembly process was characterized by surface plasmon resonance and electrochemical impedance spectroscopy. The determination of choline chloride was monitored by sensitive square wave voltammetry.

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2. Materials and methods

2.1. Chemicals

Cysteamine hydrochloride (CH) was purchased from Fluka. Glutaraldehyde (GA, 25% aqueous solution) was supplied by Acros. Chitosan was obtained from Generay Biotech Co., Ltd. Choline oxidase from alcaligenes species (ChOx, 12 units mg^{-1}) was provided by Sigma. Choline chloride (98%) was purchased from Alfa Aesar. Ascorbic acid was provided by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. Uric acid was obtained from Bio Basic INC. All other chemicals were of analytical grade and were used as received. The water used throughout the experiment was purified by a Milli-Q water system (resistivity 18.25 MΩ cm, Millipore).

2.2. Instrumentation

Transmission electronic microscopy (TEM) images were obtained by a JEOL 2000 transmission electron microscope (JEOL, Japan) operated at 200 kV. The sample for TEM characterization was prepared by evaporating a droplet of dilute solution onto carbon-coated copper grids (200 mesh). Surface plasmon resonance (SPR) and electrochemical impedance spectroscopy (EIS) experiments were carried out with an Autolab instrument (Eco Chemie B.V., Netherlands). Electrochemical experiments were performed using a conventional three-electrode system. A bare or modified gold electrode served as a working electrode, a platinum plate as a counter electrode, and an Ag/AgCl electrode with saturated KCl solution as a reference electrode. Cyclic voltammetry (CV) and square wave voltammetry (SWV) measurements were performed on a CHI 660B electrochemical workstation (Shanghai Chenhua Apparatus, China), and carried out in 0.1 M sodium phosphate buffer solution (PBS) at 25 °C, 10 ml working volume. Magnetic stirring was used before the CV and SWV measurements to ensure the homogeneity of the electrolyte solution.

2.3. Synthesis of chitosan modified Fe_3O_4 MNPs (CS-MNPs)

The chitosan modified Fe_3O_4 MNPs were prepared referring to a previously reported solvothermal method.[24] In brief, 0.41 g $FeCl_3$ was firstly dissolved in 20 mL of ethylene glycol under vigorous stirring. After the solution became clear, 1.8 g NaAc and 1.0 g chitosan were added with continuous stirring for 30 min. Then the mixture was sealed in a teflon-lined stainless-steel autoclave, which was heated to and maintained at 200 °C for 12 h. After the reaction was completed, the autoclave was cooled to room temperature. The formed black Fe_3O_4 MNPs were collected by an external magnetic field, rinsed with ethanol three times, dried at 60 °C under vacuum and made into powder.

2.4. The assembly process for the choline biosensor

Before modification, a gold electrode (d = 2 mm) was firstly polished carefully with 1, 0.3, 0.05 μm malumina slurries, and washed ultrasonically in water. Then it was electrochemically cleaned in 0.1 M H_2SO_4 by cyclic potential scanning between −0.2 and 1.55 V until a standard cyclic voltammogram of the gold electrode was obtained. The effective area of the electrode was calculated to be about 6.3 mm^2. Finally, the electrode was sonicated with water and absolute ethanol, and dried in a nitrogen stream for use.

Scheme 1 provides an overview of the fabrication process for the choline biosensor. Firstly, the cleaned bare gold electrode was incubated in 10 mM cysteamine hydrochloride for 8 h. After washing off unbound cysteamine, the electrode was dipped into 5% glutaraldehyde for 4 h. Secondly, the glutaraldehyde modified electrode was assembled with 2 mg ml^{-1} chitosan modified Fe_3O_4 MNPs for 6 h. Then the modified electrode was immersed into 5% glutaraldehyde for 4 h. Finally, the assembled electrode was coupled with 2 mg ml^{-1} choline oxidase (ChOx) for 6 h at 25 °C. The assembly solvents were all 0.02 M PBS (pH 7.0), and each step was followed with rinsing in PBS. The as-prepared electrode was denoted as a Au/CH/GA/Fe_3O_4-MNPs/GA/ChOx modified electrode, and stored in PBS solution at 4 °C until use.

3. Results and discussion

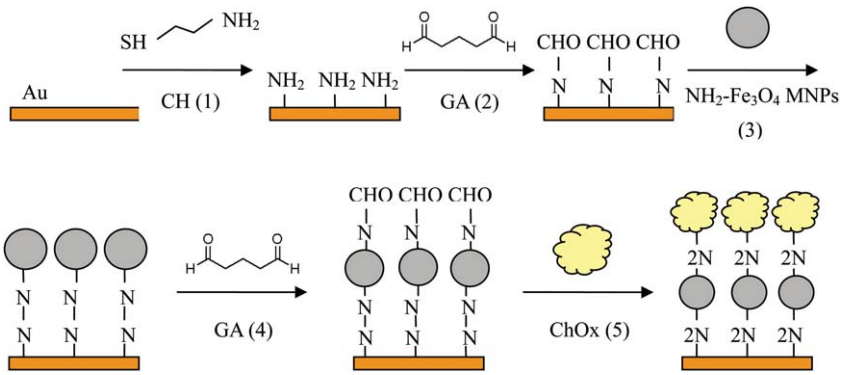
3.1. Characterization of the as-synthesised chitosan modified Fe_3O_4 MNPs (CS-MNPs)

Amino groups were introduced to the Fe_3O_4 MNPs by using chitosan *via* the solvothermal method. TEM images of the CS-MNPs are shown in Fig. 1. We can observe that the as-prepared Fe_3O_4 MNPs were well dispersed in aqueous solution, and there is an obvious chitosan layer on the surface of the Fe_3O_4 MNPs. The diameter of the spherical Fe_3O_4 MNPs is about 250 nm, and the thickness of the chitosan layer is roughly 20 nm. The other characterizations of the chitosan protected Fe_3O_4 MNPs are dipicted in Fig. 1 in our previous report.[25] Amino groups on the surface of the Fe_3O_4 MNPs are used not only as covalently linked groups, but also as protective groups. So the as-prepared Fe_3O_4 MNPs have favorable monodispersed and superparamagnetic properties in aqueous solution.

3.2. SPR and EIS characterizations of the assembly process

Surface plasmon resonance (SPR) experiments were performed to track the fabrication procedure of the choline biosensor because of its gold sensing surface. For the reflection spectrum, the measured $\Delta\theta$ value corresponds to the quality of each assembly layer with a mass sensitivity coefficient of 120 millidegrees per 100 ng cm^{-2}. As shown in Fig. 2, the cysteamine, the first glutaraldehyde, the Fe_3O_4 MNPs, the second glutaraldehyde and the choline oxidase layers caused right shifts in the SPR angles ($\Delta\theta$) by approximately 280, 210, 640, 220, 590 millidegrees, respectively. According to the mass sensitivity coefficient, the $\Delta\theta$ value of choline oxidase is 590 millidegrees, which corresponds to the surface coverage of 492 ng cm^{-2}.

Electrochemical impedance spectroscopy (EIS) was also carried out to characterize the assembly process of various organic layers on the surface of a gold electrode. 5 mM $[Fe(CN)_6]^{3-/4-}$ in 0.02 M PBS (pH 7.0) containing 0.1 M KCl was used as the electrolyte solution during the EIS measurement. As depicted in Fig. 3A, a modified equivalent circuit model was used to fit the impedance spectra.[26] The parameters in the equivalent circuit include the solution resistance (R_s), the Warburg impedance (Z_w) resulting from the diffusion of the $[Fe(CN)_6]^{3-/4-}$ redox probe, the double layer capacitance (C_d) (it may be also



Scheme 1 The fabrication process of the choline biosensor on a gold substrate. (1) The cleaned gold substrate is incubated in 10 mM cysteamine hydrochloride for 8 h. (2) The electrode is then dipped in 5% glutaraldehyde for 4 h, (3) 2 mg ml^{-1} of chitosan modified Fe_3O_4 MNPs for 6 h, (4) 5% glutaraldehyde for 4 h, and (5) 2 mg ml^{-1} of choline oxidase for 6 h. The solvents were all 0.02 M PBS (pH 7.0). CH: cysteamine hydrochloride; GA: glutaraldehyde; ChOx: choline oxidase.

substituted by the constant phase element (Q) when taking into account electrode roughness), and the charge-transfer resistance (R_{ct}). The latter two components (Q and R_{ct}) represent the interfacial properties of the electrode, which are highly sensitive to the surface texture. As shown in Fig. 3B, the bare gold electrode exhibited a small semicircle domain (curve a), corresponding to the R_{ct} value of 0.864 kΩ. When cysteamine and glutaraldehyde were assembled on the electrode surface (curve b), the R_{ct} value decreased to 0.145 kΩ. This is attributed to the electrostatic attraction between the positive charges of the cysteamine molecule and the negatively charged $[Fe(CN)_6]^{3-/4-}$ redox couple. Subsequently, Fe_3O_4 MNPs and glutaraldehyde were immobilized onto the modified electrode (curve c), and the R_{ct} value increased to 4.84 kΩ, due to the kinetics of the electron transfer restricted by the assembly layer. When the choline oxidase layer was deposited (curve d), the R_{ct} value increased to 10.66 kΩ, because of the compact structure of fabricated multilayer films. The SPR and the EIS data indicated that various organic layers were successfully assembled onto the gold substrate, and the construction of the choline biosensor on a gold electrode is feasible.

3.3. Cyclic voltammetry (CV) of the electrode with and without Fe_3O_4 MNPs

For the verification of the catalytic properties of the Fe_3O_4 MNPs, electrodes without and with the modification of Fe_3O_4 MNPs were prepared and used for the detection of 0.1 mM

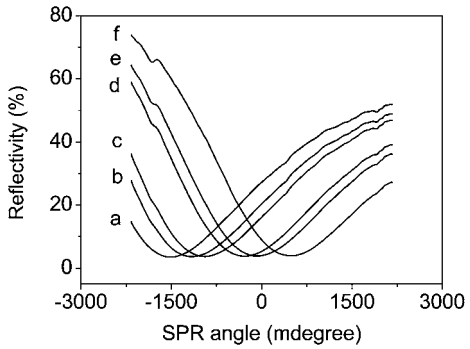


Fig. 2 The surface plasmon resonance (SPR) reflection spectrum of the assembly process. (a) Cysteamine hydrochloride; (b) glutaraldehyde; (c) Fe_3O_4 MNPs; (d) glutaraldehyde; (e) choline oxidase. The buffer 0.02 M PBS (pH 7.0) was used all the time during the SPR measurement.

choline chloride in 0.1 M PBS (pH 7.0). As depicted in Fig. 4, the Fe_3O_4 MNPs modified electrode has a much higher reduction peak of H_2O_2 than the electrode without the assembly of Fe_3O_4 MNPs at −0.2 V. Moreover, the Fe_3O_4 MNPs modified electrode has two apparent oxidation and reduction peaks at 0.42 V and 0.37 V, respectively. This phenomenon could be interpreted in Scheme 2. When performing the positive scan from −0.2 V to 0.8 V, choline was catalyzed by choline oxidase and oxidized to produce betaine aldehyde and H_2O_2. So the oxidation peak at 0.42 V is the oxidation peak of choline oxidase. While in the process of the negative scan, the generated H_2O_2 was catalyzed

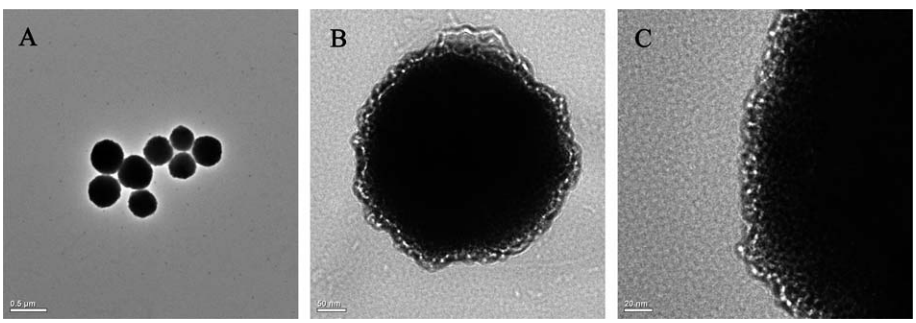


Fig. 1 Transmission electronic microscopy (TEM) images of chitosan-modified Fe_3O_4 MNPs.

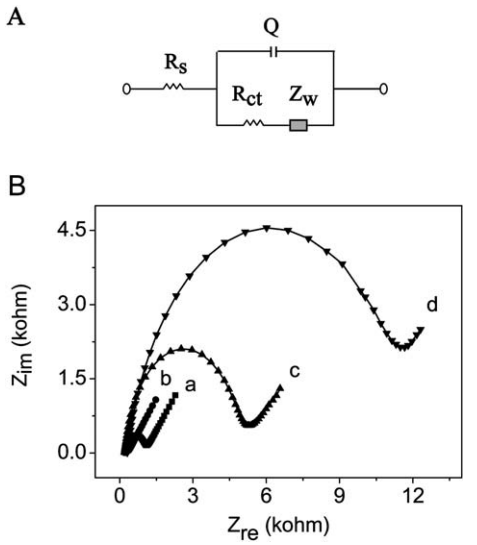


Fig. 3 (A) The Randle modified equivalent circuit model for the impedance spectra. (B) The Nyquist electrochemical impedance spectroscopy (EIS) of the modification procedure on a gold electrode in the presence of 5 mM $[Fe(CN)_6]^{3-/4-}$ in 0.02 M PBS (pH 7.0) containing 0.1 M KCl. (a) Bare gold electrode; (b) cysteamine hydrochloride and glutaraldehyde; (c) Fe_3O_4 MNPs and glutaraldehyde; (d) choline oxidase. AC potential, 0.23 V; frequency range, 100 KHz–0.1 Hz; voltage amplitude, 5 mV.

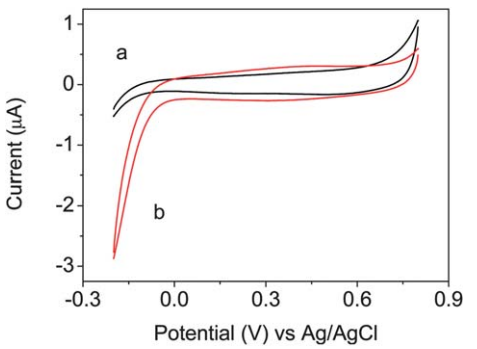


Fig. 4 The cyclic voltammogram (CV) of (a) the electrode without (Au/CH/GA/ChOx) and (b) with modification of Fe_3O_4 MNPs (Au/CH/GA/Fe_3O_4-MNPs/GA/ChOx) in the presence of 0.1 mM choline chloride in 0.1 M PBS (pH 7.0), scan rate 50 mV s^{-1}.

by Fe_3O_4 MNPs, and reduced to produce H_2O. Therefore, the reduction peak at 0.37 V corresponds to the reduction peak of Fe_3O_4 MNPs. Through the comparison of the electrode with and without Fe_3O_4 MNPs using the CV scan, it was found that the electrode with the modification of Fe_3O_4 MNPs has a relatively high signal response when detecting the analyte choline chloride. It has been reported that each Fe_3O_4 MNP we used in this paper is a cluster of many smaller crystals with diameters varying from 10 to 20 nm rather than one single crystal structure.[24] We know that, in general, the smaller the nanoparticles are, the higher catalytic activity they will possess. This phenomenon may be due to the smaller nanoparticles having a greater surface-to-volume to interact with the substrates. Therefore, we can infer that the Fe_3O_4 MNPs used here as peroxidase mimics have a relatively high catalytic property.

We can explain the catalytic reaction mechanism of Fe_3O_4 MNPs according to the literature.[10] (1) Fe_3O_4 MNPs have peroxidase-like activity towards typical peroxidase substrates. (2) However, through the Michaelis constant (k_m) experiments, we found that the catalytic activity of Fe_3O_4 MNPs with H_2O_2 as the substrate was lower than that for HRP. (3) Fe^{2+} ions may play a dominant role in the catalytic peroxidase-like activity of Fe_3O_4 MNPs. (4) Fe_3O_4 MNPs may perform a ping-pong mechanism, as is observed for HRP. This then indicates that, as for HRP, the MNPs bind and react with the first substrate, releasing the first product before reacting with the second substrate.[27]

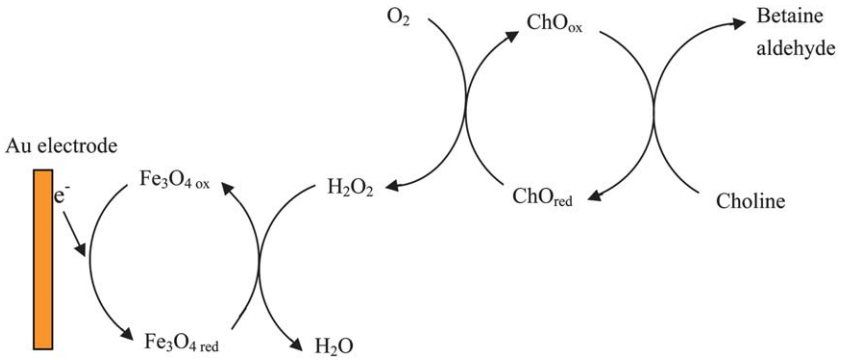
3.4. Effect of pH value and temperature on the response of the choline biosensor

It was reported that the catalytic activity of Fe_3O_4 MNPs and ChOx is dependent on pH and temperature.[10,17] Thus, the effects of the pH value and temperature of the detection solution on the response behavior of the biosensor were investigated in the current study. In order to reduce the impact of some interfering substances, the reduction current of square wave voltammetry at the scanning potential range from −0.2 V to 0.8 V was used as the detection signal. As shown in Fig. 5, the effect of pH value on the electrode response was investigated in the pH range 4.0–10.0 in the presence of 0.1 mM choline chloride. It shows that the current response of the modified biosensor is enhanced with increasing pH value. When the pH value is approximately equal to 8.0, the current response reaches a maximum, and then goes down as the pH value turns higher. This phenomenon may be attributed to the ChOx being denatured at lower or higher pH values. Thus, pH 8.0 was selected as optimum for further experiments.

Fig. 6 shows the effect of temperature on the performance of the biosensor. The temperature is stepped from 25 to 60 °C in 0.1 mM choline chloride. The current response increases slowly with the temperature from 25 to 40 °C, and then decreases sharply from 0.4 to 0.6 V. Therefore, 40 °C was selected as the optimum temperature.

3.5. Determination of choline chloride using square wave voltammetry (SWV)

Ascorbic acid (AA) and uric acid (UA) may interfere in the determination of choline chloride because of their electrochemical activities and coexistence in organisms.[17] Through our preliminary experiment, we found that AA and UA have significant oxidation peaks separately at 0.2 V and 0.5 V. For decreasing the interference of AA and UA, the reduction current of the SWV was used to determine choline chloride. As shown in Fig. 7A, a typical SWV scanning of the modified electrode from 0.8 to −0.2 V in 0.1 M PBS (pH 8.0) at 40 °C with different concentrations of choline chloride was obtained. The reduction currents at 0.37 V gradually increase when increasing the concentrations of choline chloride. A positive linear relationship between the reduction currents and the logarithm values of choline concentrations in the range from 10^{-9} to 10^{-2} M is depicted in Fig. 7B. The regression equation is $Y = 2.56 + 0.22X$ (Y: the reduction current, μA; X: the logarithm value of choline concentration, M), with a correlation coefficient (R) of 0.995 ($n =$



Scheme 2 The reaction illustration of the Au/CH/GA/Fe_3O_4-MNPs/GA/ChOx modified electrode for the detection of choline chloride in aqueous solution.

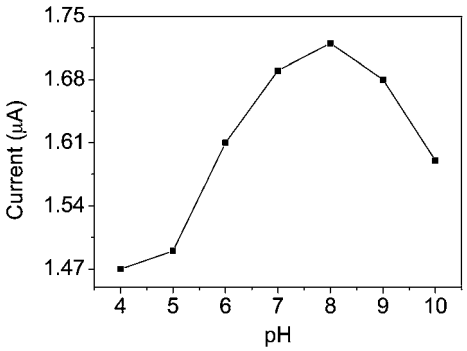


Fig. 5 Effect of pH value on the current response of the biosensor to 0.1 mM choline chloride obtained from the reduction current of square wave voltammetry (SWV) at the scanning potential range from −0.2 V to 0.8 V.

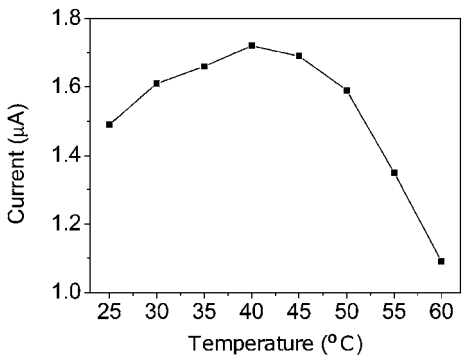


Fig. 6 Effect of temperature on the response of the biosensor to 0.1 mM choline chloride, using the reduction current of SWV at the scanning potential range from −0.2 V to 0.8 V as the detection signal.

3). In addition, the detection limit of 0.1 nM (S/N = 3) was estimated for the choline biosensor. These data suggested that such a choline biosensor is relatively more sensitive than some available choline biosensors.[17–19,28]

3.6. Selectivity, reproducibility and stability of the choline biosensor

AA and UA were used as the interference substances for the selectivity experiments. AA solution was prepared with 0.02 M PBS (pH 7.0). While UA was firstly dissolved in 0.1 M NaOH and diluted to a certain concentration using 0.02 M PBS (pH 7.0). The reduction currents of SWV increased only 5.8% and 3.9% for 0.05 mM choline chloride after adding 0.05 mM AA and 0.01 mM UA, respectively. The reason is probably that, although AA and UA have high electrochemical activities on the oxidation process in our pre-experiments, their reduction currents are relatively small and can be neglected according to the literature.[17,29,30] Therefore, the interferences of AA and UA are very small when using the reduction currents of SWV for the determination of choline chloride.

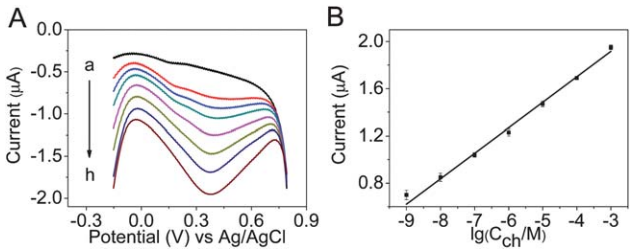


Fig. 7 Square wave voltammogram (SWV) of the modified electrode in 0.1 M PBS (pH 8.0) at 40 °C with different concentrations of choline chloride. From the top down: 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} M. (B) The corresponding calibration curve of the choline biosensor at 0.37 V ($n = 3$).

The reproducibility of the choline biosensor was measured in 0.1 M PBS (pH 8.0) at 40 °C. The relative standard deviation (RSD) of the modified electrode response to 1 μM choline chloride was 6.7% for ten successive measurements. For the stability text, the choline biosensor was stored in 0.02 M PBS (pH 7.0) at 4 °C and measured at intervals of three days. It retained about 85% of its original response after one month. These data suggested that the choline biosensor is provided with relatively high reproducibility and stability. These can perhaps be attributed to the stability of the choline biosensor being mainly dependent upon the ChOx stability, because the stability of Fe_3O_4 MNPs is relatively high under the optimal experimental conditions.

4. Conclusions

In this work, a sensitive choline biosensor using a Fe_3O_4 MNPs and choline oxidase modified gold electrode was fabricated. When comparing the electrode without the assembly of Fe_3O_4

MNPs, the Fe_3O_4 MNPs modified electrode has a better signal response in choline chloride detection. Moreover, Fe_3O_4 MNPs as peroxidase mimics to catalyze the reduction of H_2O_2 have some merits over natural peroxidases (HRP, *etc.*), such as higher stability, properties of magnetic separation and enrichment, and easy preparation, *etc.* The choline biosensor exhibited favorable sensitivity, selectivity and stability in the detection of choline chloride. This work using Fe_3O_4 MNPs as peroxidase mimics to improve the signal responses of the choline biosensor, establishes a methodology for the developing of nanomaterial-functional biosensors with good analytical properties in the future.

Acknowledgements

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