



A sensitive choline biosensor with supramolecular architecture

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ABSTRACT

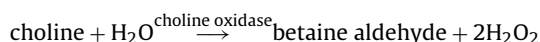
A sensitive biosensor with supramolecular architecture was designed and implemented here to detect choline. Choline oxidase and horseradish peroxidase were assembled onto the polymer of thiolated β -cyclodextrin and platinum nanoparticles modified gold electrode through 1-adamantane carboxylic acid coupling. Square wave voltammetry showed that the reduction currents at 0.38 mV had a linear relationship with the logarithms of choline concentrations in the range of 10^{-9} – 10^{-2} M, and the detection limit was down to 0.1 nM. Such biosensor also exhibited excellent selectivity, reproducibility and stability.

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1. Introduction

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 membranes [1]. Choline deficiency can affect memory and easily cause liver dysfunction or cancer, and abnormal choline metabolism has been implicated in a number of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [2,3]. Therefore, Academy of Pediatrics, Food and Nutrition Board in some countries have established an intake of choline in infants and adults every day, respectively [4].

Lately, enzyme-based biosensors have emerged for the direct monitoring of choline. The single enzyme (choline oxidase) or bi-enzyme (choline oxidase/horseradish peroxidase) electrodes were assembled for the determination of choline based on the detection of liberated hydrogen peroxide [5–7]. In the presence of oxygen, choline is catalyzed by choline oxidase and converted into hydrogen peroxide. In the comparison of single enzyme and bi-enzyme choline biosensors, the bi-enzyme system has the high sensitivity and the low detection limit according to the literature [8]. So the bi-enzyme choline biosensor attracts more attention.



The strategies for enzyme immobilization are critical in various enzyme biosensors. Nowadays, many enzyme biosensors have been developed based on the physical adsorption of glass carbon or carbon paste electrodes (mainly graphite) [9,10]. However, the reproducibilities of these biosensors are not so satisfactory [11,12]. Covalent immobilization is another common method for the construction of various biosensors. While the fabrication process is readily to inactivate the biomolecules on the electrode surface, because some chemical reagents are used as connecting agents, such as glutaraldehyde, etc. [13]. More recently, supramolecular association has attracted considerable attention, since the host molecules are three-dimensional matrices and the bioactivity of various organisms on biosensors can be effectively retained in the immobilization and reaction processes [14–16]. The most common host molecules are various cyclodextrins (CDs). They are a class of cyclic oligosaccharides with a hydrophobic inner cavity and a hydrophilic outside surface, and can form stable inclusion complexes with organic molecules through hydrophobic interaction [17,18]. Lately, Villalonga's group has developed a xanthine biosensor with supramolecular architecture [19]. But to date there have been no report about the choline biosensor via supramolecular associations.

In this paper, a sensitive choline biosensor was constructed with supramolecular architecture. Thiolated β -cyclodextrin was firstly synthesized. It was polymerized with Pt nanoparticles, which have been widely used in various biosensors due to the biocompatibility and good electrocatalytic activity to H_2O_2 [20,21]. Then the polymer of thiolated β -cyclodextrin and Pt nanoparticles was immobilized onto a gold electrode through thiolate-gold bonds. Finally, the modified electrode was connected with 1-adamantane

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carboxylic acid, which could crosslink with choline oxidase and horseradish peroxidase. The whole assembly process was characterized by electrochemical impedance spectroscopy and surface plasmon resonance, and the determination of choline was monitored by sensitive square wave voltammetry.

2. Experimental

2.1. Reagents

β -Cyclodextrin (β -CD) was purchased from Tianjin Damao Chemical Reagent Factory. Hydrogen hexachloroplatinate (IV) hydrate ($\text{H}_2\text{PtCl}_6 \cdot \text{H}_2\text{O}$) was supplied by Shanghai Sinopharm Chemical Reagent Co., Ltd. 1-Adamantane carboxylic acid was obtained from Aldrich. Horseradish peroxidase (HRP, 300 units/mg) was provided by Beijing Dingguo Biotechnology Co., Ltd. Choline oxidase from *Alcaligenes species* (ChOx, 12 units/mg) was obtained from Sigma. Choline chloride (98%) was supplied by Alfa Aesar. Ascorbic acid was purchased from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. Uric acid was provided by Bio Basic INC. All other chemicals were of analytical grade and were used as received. Milli-Q water (resistivity, 18.25 M Ω cm) was used throughout.

2.2. Apparatus

Electrochemical experiments were carried out using a conventional three-electrode system. A bare or modified gold electrode was served as a working electrode, a platinum wire 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, pH 7.0) at 25 °C, 10 ml working volume. Magnetic stirring was used before CV and SWV measurements to ensure the homogeneity of the solution. Electrochemical impedance spectroscopy (EIS) and surface plasmon resonance (SPR) experiments were carried out with an Autolab instrument (Eco Chemie B.V., Netherlands). X-ray photoelectron spectroscopy (XPS) analysis was conducted with an ESCLAB MK II spectrometer (VG Co., UK) using Mg as the exciting source. Pt and S concentrations of the polymer solution of Pt nanoparticles and SH- β CD were determined using an iCAP 6000 inductively coupled plasma optical emission spectroscopy spectrometer (ICPOES) (Thermo).

2.3. Preparation of the polymer of Pt nanoparticles and thiolated β -cyclodextrin

Thiolated β -cyclodextrin (SH- β CD, MW 1247) was synthesized according to the literature [22]. The seven primary hydroxyl groups of β -CD were replaced by thiol groups. Pt nanoparticles were synthesized by the method of Willner's group [23]. The diameters of as-prepared Pt nanoparticles were about 2 nm through transmission electron microscopy (TEM). 2 mg, 0.2 mg and 0.02 mg of SH- β CD were respectively dissolved in 200 μ L of dimethyl sulfoxide (DMSO), followed by adding 800 μ L of Pt nanoparticles solution, and then the mixture was stirred for 24 h. The choline biosensors with different amount of SH- β CD were used to detect 1 mM choline chloride. Through the reduction currents of SWV, we found 0.2 mg of SH- β CD has the better result. So 0.2 mg of SH- β CD was selected for the subsequent experiments (data not shown). The obtained polymer was labeled as PtpolyCD.

The Pt nanoparticles in the polymer solution were centrifugated at 16,000 rpm for 20 min, then the precipitate was successively washed twice with water through centrifugation. Finally, the Pt

nanoparticles were dispersed in the same volume of water for iCAP experiment.

A gold substrate was cleaned in piranha solution ($\text{VH}_2\text{SO}_4/\text{VH}_2\text{O}_2 = 3:1$). Then the cleaned gold plate was incubated in the PtpolyCD solution for 12 h, washed with water, and dried in air for XPS experiment.

2.4. Preparation of the enzyme solution

6 mg of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC), 1 mg of N-hydroxysuccinimide (NHS), 2 mg of HRP and 2 mg of ChOx were dispersed in 1 mL of 0.02 M PBS (pH 6.0) and shaken for 4 h. The concentration ratio of HRP and ChOx was referred to the report [8]. Then the mixed enzyme solution was kept at 4 °C before use.

2.5. 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 μ m alumina slurries, and sonicated 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 gold electrode was obtained. The effective area of the electrode was calculated to be about 6.4 mm². Finally, the electrode was sonicated in water and absolute ethanol, and dried in a nitrogen stream.

Scheme 1 provides an overview of the fabrication process for the choline biosensor. First, the cleaned bare gold electrode was dipped into the PtpolyCD solution overnight, followed by rinsing with absolute ethanol and water. The PtpolyCD modified electrode was further immersed into a 2 mg/mL of 1-adamantane (1-ADA) carboxylic acid solution in 0.02 M PBS (pH 7.0) for 24 h, and washed with water. Finally, the bi-enzyme ChOx and HRP were cross-linked with 1-ADA carboxylic acid by dipping the modified electrode in the mixed enzyme solution (section 2.4) at room temperature. After 24 h incubation, the electrode was washed with 0.02 M PBS (pH 7.0) and kept at 4 °C until use. The bi-enzymes were assembled onto the electrode surface via a carbodiimide-catalyzed reaction. The as-prepared electrode was denoted as Au/PtpolyCD/ADA/E.

3. Results and discussion

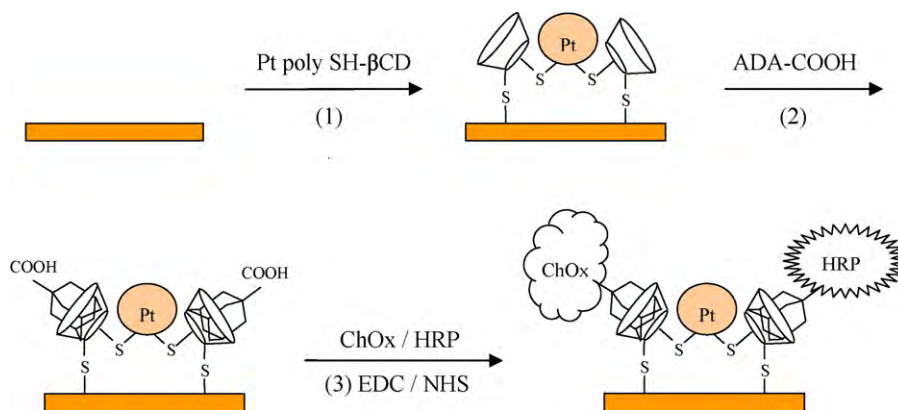
3.1. XPS characterization of PtpolyCD

Pt and S concentrations of the polymer solution of Pt nanoparticles and SH- β CD were 0.6695 and 3.445 ppm through iCAP experiment. There are seven S atoms in each β -CD molecule. The ratio of the Pt nanoparticles and the SH- β CD in the PtpolyCD solution was an average of 15.38 mmol of SH- β CD per 21 μ mol of Pt nanoparticles.

XPS data were collected to confirm the Pt nanoparticles were assembled onto the bare gold substrate. Distinct Pt (4f7/2) and Pt (4f5/2) peaks at 73.3 eV and 75.7 eV are observed in Fig. 1, respectively. These values lie between zero-valent Pt (4f7/2 71.3 eV, 4f5/2 74.5 eV) and two-valent Pt (4f7/2 73.5 eV, 4f5/2 76.8 eV) [24]. The Pt nanoparticles in our experiment should be zero-valent according to the literature [25]. This may be attributed to the small particle size (2 nm) and high activity, and partial Pt was oxidized into two-valent Pt ions. The result confirmed not only the formation of the polymer of Pt nanoparticles and SH- β CD, but also the successful immobilization of the PtpolyCD polymer.

3.2. EIS and SPR characterizations of the assembly process

SPR experiments were performed to track the mass changes of the assembly layers. As shown in Fig. 2A, when the PtpolyCD layer



Scheme 1. The assembly process on the gold substrate. (1) The PtpolyCD solution for 12 h; (2) the 2 mg/mL of 1-adamantane carboxylic acid solution for 24 h; (3) the enzyme solution for 24 h at room temperature.

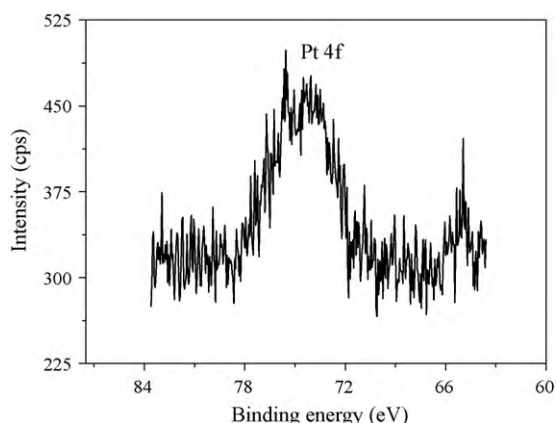


Fig. 1. XPS spectra of Pt in the as-prepared PtpolyCD polymer.

was assembled on the bare gold surface (curve b), the SPR angle shifted to the right by approximately 450 millidegree. The sequential immobilization of the adamantane layer (curve c) led to positive shift in the SPR angle by about 300 millidegree. As expected, the deposition of the enzyme layer (curve d) caused right shift in the SPR angle by approximately 680 millidegree.

As depicted in Fig. 2B insert, a modified equivalent circuit model [26] was used to fit impedance spectrums. The parameters in the equivalent circuit include the solution resistance (R_s), the Warburg impedance (Z_w) resulting from the diffusion of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe, the double layer capacitance (C_d) (it may be also 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 interfacial properties of the electrode, which are highly sensitive to the surface modification. As shown in Fig. 2B, the bare gold electrode exhibited a very small semicircle domain (curve a), the R_{ct} value was only 230 Ω . When the PtpolyCD polymer was immobilized on the electrode surface (curve b), the R_{ct} value increased to about 1.6 k Ω , due to the kinetics of the electron transfer restricted by the assembly layer. The subsequent deposition of the adamantane layer (curve c), the R_{ct} value increased to approximate 3.1 k Ω . This was attributed to the electrostatic repulsion between the negative charges of adamantane molecules and the negatively charged $[\text{Fe}(\text{CN})_6]^{3-/4-}$ probe. The R_{ct} value increased to about 4.6 k Ω during further assembly of the enzyme layer (curve d). The SPR and EIS data indicated that various organic layers were successfully assembled onto the gold substrate, and the successful construction of the choline biosensor on the gold electrode.

3.3. CV behavior of choline chloride on the Au/PtpolyCD/ADA/E electrode

To decrease the overpotential and the sensitivity for choline detection, we immobilized two enzymes, HRP and ChOx, on the modified electrode surface. Fig. 3 presented the CV of the modified electrode in the presence of choline chloride at different concentrations. No obvious oxidation and reduction peaks were observed in the absence of choline chloride (curve a). Upon the addition of choline chloride, the oxidation and reduction peak (0.42 V and 0.38 V) currents increased with higher choline concentrations (curve c and d). These phenomena could be interpreted in

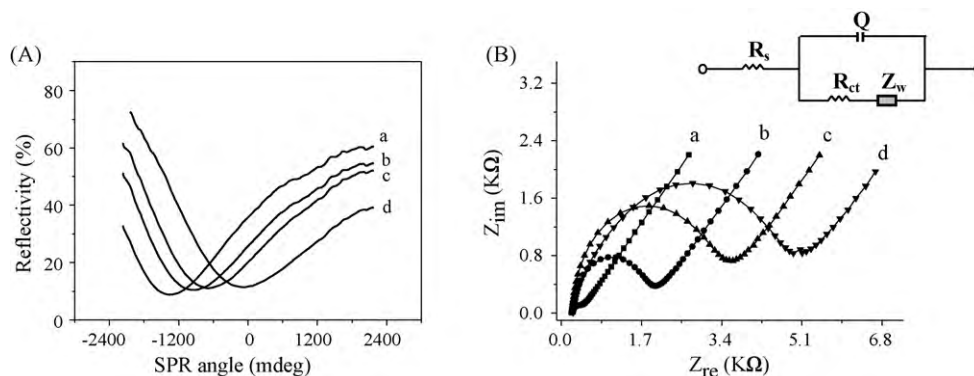
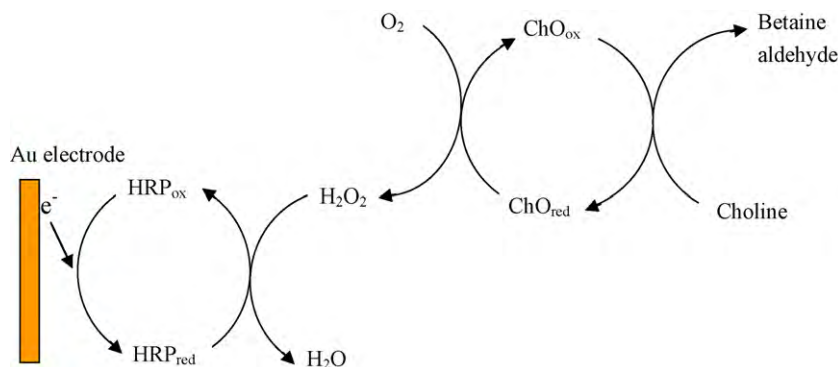


Fig. 2. (A) SPR curves of the modification process. (a) Au; (b) Au/PtpolyCD; (c) Au/PtpolyCD/ADA; (d) Au/PtpolyCD/ADA/E. (B) The EIS of the assembly process in the presence of 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 0.02 M PBS (pH 7.0) containing 0.1 M KCl. (a)–(d) as in figure 2A. Insert: circuit for the EIS.



Scheme 2. The reaction illustration of the Au/PtpolyCD/ADA/E electrode for the detection of choline in solution.

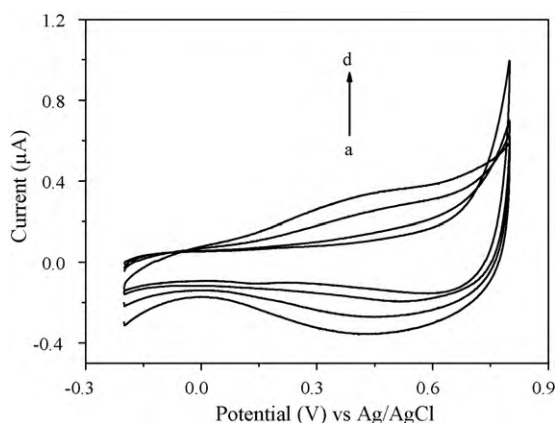


Fig. 3. CV of the Au/PtpolyCD/ADA/E electrode at 50 mV/s in 0.1 M PBS (pH 7.0), in the absence (a) and the presence of 0.1 mM (c) and 1 mM (d) choline chloride, (b) after incubation in saturated 1-Adamantane carboxylic acid solution for 24 h, the electrode in the presence of 1 mM choline chloride.

Scheme 2. In the process of positive scan, choline was catalyzed by ChOx, and oxidized to produce H_2O_2 . So the oxidation peak at 0.42 V is corresponding to the oxidation peak of ChOx. When performing the negative scan, the H_2O_2 was catalyzed by HRP, and reduction to produce H_2O . Therefore, the reduction current at 0.38 V is also the reduction of HRP. The overpotential of the modified electrode for the reduction of H_2O_2 is effectively decreased. The reason probably is that the three-dimensional structure of the host molecule and can protect the organisms on the electrode surface from damage in the reaction process. According to the above interpretation, we have no difficulty to understand the oxidation and reduction peak currents increased with the increase of choline concentrations.

In order to confirm the supramolecular mechanism between SH- β CD and 1-Adamantane carboxylic acid, the Au/PtpolyCD/ADA/E electrode was incubated in the saturated 1-Adamantane carboxylic acid solution in 0.02 M PBS (pH 7.0) for 1 day at 4 °C. The significant decrease of currents in the presence of 1 mM choline chloride could be observed in Fig. 3 (curve b). It is clear that 1-Adamantane derivatives can form stable inclusion complexes with CDs [27], so the saturated 1-Adamantane carboxylic acid solution should disrupt the host-guest interactions, resulting in the release of enzymes from the electrode surface [19].

3.4. Comparison the non-Pt nanoparticles modified electrode with Pt nanoparticles modified electrode through the SWV experiment

As depicted in Fig. 4, through comparing the non-Pt nanoparticles modified electrode with the Pt nanoparticles modified electrode, we found that the Pt nanoparticles modified electrode

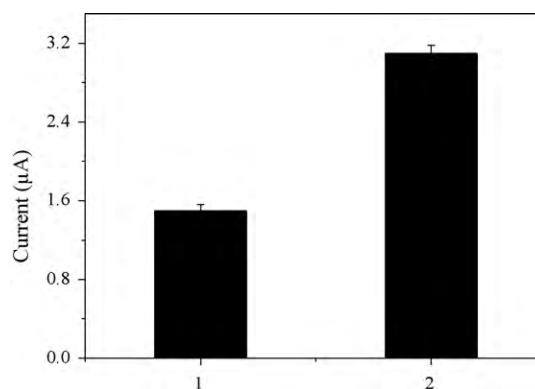


Fig. 4. The modified electrode without (1) and with Pt nanoparticles (2) for the determination of 1 mM choline chloride in 0.1 M PBS (pH 7.0) using the reduction current of SWV ($n=3$).

has the higher SWV signal in the presence of 1 mM choline chloride, which was about 2-fold enhanced by the presence of the Pt nanoparticles. The result confirmed that the Pt nanoparticles on the modified electrode not only facilitated the electron transport chain but also might accelerate the catalytic reduction process of H_2O_2 .

3.5. SWV experiments for the determination of choline chloride

Fig. 5A showed a typical SWV scanning from 0.8 to -0.2 V in 0.1 M PBS (pH 7.0) with different concentrations of choline chloride at the modified electrode. The reduction currents gradually increase with the concentrations of choline chloride. A positive linear relationship between the reduction currents and the logarithms of choline concentrations in the range from 10^{-9} to 10^{-2} M was observed in Fig. 5B. The regression equation was $Y=4.26+0.35X$ (Y : the reduction current, μA ; X : the logarithm of choline concentration, M), with a correlation coefficient (R) of 0.998 ($n=3$). In addition, a low detection limit of 0.1 nM ($S/N=3$) was estimated for this biosensor, which was more sensitive than those of most available choline biosensors [28,6].

3.6. Selectivity, reproducibility and stability of the choline biosensor

Ascorbic acid (AA) and uric acid (UA) may interfere in the determination of choline chloride because of their electrochemical activities and coexistence in organisms [4]. So we used AA and UA as the interference materials for the selectivity experiment. The AA solution was prepared with 0.02 M PBS (pH 7.0), then adding

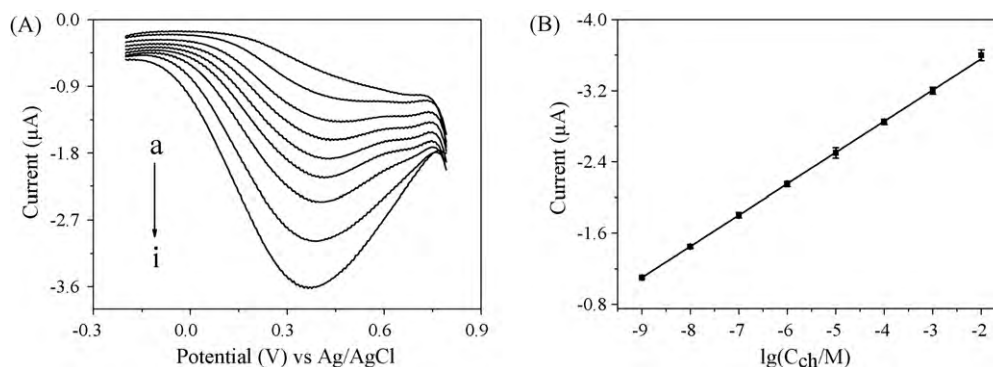


Fig. 5. (A) SWV of the Au/PtpolyCD/ADA/E electrode in 0.1 M PBS (pH 7.0) at different concentrations of choline chloride, from the top down: 0, 10^{-9} , 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} , 10^{-2} M. (B) The corresponding calibration curve of the choline biosensor at 0.38 V ($n=3$).

into 0.1 M PBS (pH 7.0) electrolyte solution for the SWV experiment. UA was firstly dissolved in 0.1 M NaOH, then using 0.02 M PBS (pH 7.0) to dilute into a certain concentration as were used in AA. The reduction currents increased only 5.2% and 4.3% for 0.05 mM choline chloride after adding 0.05 mM AA and 0.01 mM UA, respectively. The reason probably is that, although AA and UA separately have the high electrochemical activities at about 0.2 V and 0.5 V on the oxidation process in our previous experiments. Their reduction currents are very small and can be neglected according to the literatures [4,29,30]. Therefore, when using the reduction currents of SWV for the determination of choline concentrations, the influences of AA and UA are relatively small. The relative standard deviation (RSD) of ten successive measurements in the presence of 1 μ M choline chloride was 4.6%. The modified electrode was stored in 0.02 M PBS (pH 7.0) at 4 °C and measured at intervals of one week. It remained about 85% of its original response after one month. The results clearly showed the strategy proposed here offered the advantages of selectivity, reproducibility and stability towards the detection of choline.

4. Conclusion

In this work, a choline biosensor based on the Au/PtpolyCD/ADA/E modified electrode was constructed. It exhibited high sensitivity, favorable selectivity and stability in the detection of choline chloride. The as-prepared biosensor has several advantages. (1) Through supramolecular immobilization, the enzymatic activity can be effectively retained according to literatures. (2) The response signals of the modified electrode are enhanced by the presence of Pt nanoparticles. (3) Using the reduction currents of sensitive SWV technique as the detection signals, not only improve the sensitivity of the choline biosensor, but also decrease the interference of AA and UA. This work establishes a methodology for the fabricating of biosensors with good analytical properties.

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