Talanta 80 (2009) 484–488

Contents lists available at ScienceDirect

Talanta

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Electrochemical determination of melamine using oligonucleotides modified gold electrodes

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ARTICLE INFO

Article history:
Received 7 May 2009
Received in revised form 30 June 2009
Accepted 2 July 2009
Available online 10 July 2009

Keywords:
Oligonucleotides
Melamine
Differential pulse stripping voltammetry
Electrochemical impedance spectrometry

ABSTRACT

A novel and simple electrochemical method for determination of melamine is developed based on oligonucleotides film modified gold electrodes. The electrochemical probe of ferricyanide was used to investigate the interactions between oligonucleotides and melamine. Results of cyclic voltammetries, differential pulse stripping voltammetries, electrochemical impedance spectrometry and atomic force microscope, proved that melamine might interact with oligonucleotides mainly through electrostatic and hydrogen-bonding interactions. The interactions between oligonucleotides and melamine lead to the increase in the peak currents of ferricyanide, which could be used for electrochemical sensing of melamine. The redox peak currents of ferricyanide were linear with the concentration of melamine in the range from 3.9×10^{-8} to 3.3×10^{-6} M with a linear coefficiency of 0.990. The detection limit was 9.6×10^{-9} M. The proposed electrochemical biosensor is rapid, convenient and low-cost for effective sensing of melamine. Particularly, the proposed method was applied successfully to the determination of melamine in milk products, and the recovery was 95%.

1. Introduction

Melamine, a kind of triazine analogue with three amino groups (Fig. 1), is widely used as an industrial chemical in the production of melamine resins, which are used in laminates, glues, adhesives, and plastics. Melamine can be found as a contaminant in a variety of foods from food-contact materials. When added to milk powder, melamine increases the nitrogen concentration, which suggests a false increase in protein concentration. When meeting strong acids or alkali, melamine yields ammeline, ammelide and cyanuric acid by hydrolysis. Melamine could form insoluble complexes with cyanuric acid, depending on urine pH, which could lead to crystallization and subsequent tissue injury [1], such as urolithiasis and bladder cancer. Recently many young children have suffered from renal stone caused by melamine in the milk powder, so significant efforts have been made on the fast and effective detection of melamine.

Up to now, some methods, such as gas chromatography (GC) [2], high performance liquid chromatography (HPLC) [3], high performance liquid chromatography/mass spectrum (HPLC/MS) [4], surface enhanced raman spectroscopy [5] and capillary zone electrophoresis/mass spectrum (CE/MS) [6] have been employed for the determination of melamine. However, complicated preconcentration, time-consuming steps and high-cost instruments limit the application of the existing methods. Hence, there have been increasing demands for new, fast, simple, convenient and sensitive methods for the determination of melamine. Compared to those existing methods, much importance has been attached to the electrochemical sensors due to their simplicity, low-cost, accurateness, sensitivity and high stability. Unfortunately, so far few of papers have been reported concerned about electrochemical methods to determine melamine at solid electrodes.

In this paper, surface electrochemical method based on an oligonucleotide ($d(T)_{20}$) film modified electrode is developed for the determination of melamine (Fig. 2). Due to complementary triamino triazine unit, melamine can be immobilized onto the oligonucleotides modified electrode via electrostatic interaction and hydrogen-bonding [7]. The morphologic differences between melamine and melamine interacted with oligonucleotides were observed by AFM. The electrochemical probe of $[Fe(CN)_6]^{3-/4-}$ was used to investigate the interaction between melamine and oligonucleotides since melamine is non-electroactive over the potential range studied. The peak currents of the electrochemical probe evaluated by differential pulse stripping voltammetry (DPSV) were linear with the concentrations of melamine in the range from 3.9×10^{-8} to 3.3×10^{-6} M with a linear coefficiency of 0.990. The detection limit was 9.6×10^{-9} M. On the other hand, electrochemical impedance spectrometry is a useful

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doi:10.1016/j.talanta.2009.07.006

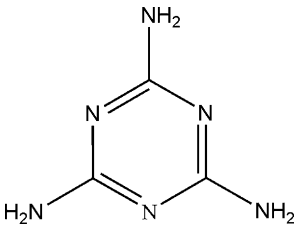

Fig. 1. The structure of melamine.

method to study the interfacial properties of modified electrode [8–12]. The immobilization of materials on the electrode surface might probably alter the interfacial electron transfer features at the electrode surface. Therefore, in this study, electrochemical impedance spectrometry was further used to study the interaction between oligonucleotides and melamine. The proposed method was applied successfully to the determination of melamine in milk products, and the recovery was 95%. To the best of our knowledge, the proposed method has not been reported, which will have great implications in the determination of melamine in food.

2. Experimental

2.1. Chemicals and materials

Melamine, analytical grade, was purchased from Sigma (USA). Oligonucleotides ($d(T)_{20}$) containing a 6-mercaptohexyl linker at the 5′ end was obtained from Shanghai Invitrogen Corporation (China). The $d(T)_{20}$ stock solution (20 μM) was prepared by dissolving the $d(T)_{20}$ powder in 0.01 M phosphate buffer solution (pH 7.0) containing 0.5 M NaCl and stored at 4 °C. Other chemicals were of analytical reagent grade and used as received. All aqueous solutions were prepared with distilled water.

2.2. Apparatus and equipments

Electrochemical measurements were carried out by a CHI 760C electrochemical workstation (CHI Instrument Company, Shanghai, China). A three-electrode system was used in the measurements, composed of a bare gold electrode (d = 2 mm) or $d(T)_{20}$/Au as the working electrode, a saturated calomel electrode (SCE) as the reference electrode and the Pt wire as the counter electrode. All the pH measurements were performed with PB-10 pH meter (Sartorius, Germany). The images of atomic force microscope (AFM) were obtained by Veeco Dimension 3100 (Digital Instrument, USA) in the tapping mode in air.

2.3. Preparation of $d(T)_{20}$ modified electrodes

Gold electrode was cleaned by exposure to warm piranha solution (70% concentrated sulfuric acid, 30% peroxide solution (30%)) for 10 min, and rinsed ultrasonically with ethanol and water. Then the electrode was cycled between 0.2 and 1.5 V in 0.1 M H_2SO_4 at 100 mV/s until reproducible voltammograms were obtained.

Self-assembled monolayer was prepared by transferring 15 μL $d(T)_{20}$ solution onto the gold electrode's surface. After the 16 h immobilization period, the electrode was soaked in sterilized water for 2 h to remove any un-adsorbed $d(T)_{20}$, and dried with a nitrogen stream. Thus, the $d(T)_{20}$ modified gold electrode($d(T)_{20}$/Au) was obtained.

2.4. Atomic force microscopy

Cleaned mica substrates were dried under a stream of N_2, and a drop of melamine phosphate buffer solution (pH = 7.0) was deposited on the freshly mica surface. Half an hour later the excess solution was gently cleaned with a jet of distilled water, and the mica with adsorbed melamine molecules was then dried in a sterile atmosphere. Afterwards a drop of $d(T)_{20}$ phosphate buffer solution (pH = 7.0) was deposited on the positively charged melamine-modified substrates for 1–4 h as incubation time, and the excess solution was gently cleaned with a jet of distilled water. After the mica substrate was completely dried, the morphology of the resulting surfaces was characterized using atomic force microscopy.

3. Results and discussion

3.1. Atomic force microscopy evaluation of melamine–$d(T)_{20}$ interaction

Atomic force microscopy is another common method to study the interaction between small molecules and oligonucleotides [13–15]. Therefore, it is used to further investigate the interaction between melamine and $d(T)_{20}$. Mica was chosen as substrate because of its atomically flat surface over a large area. A drop of melamine solution spreads rapidly across the mica surface in a thin layer because of the high hydrophilicity of the mica surface when it is deposited on a freshly cleaved mica surface. Cationic melamine can be easily adsorbed on the negatively charged mica surface by electrostatic attractive force (Fig. 3A). Firstly we changed the incubation time to evaluate the effect of incubation time on $d(T)_{20}$ immobilization. Fig. 3B and C show the AFM images of $d(T)_{20}$ molecules absorbed on the melamine-modified mica surface for 1–2 h incubation time, respectively. The $d(T)_{20}$ molecules which carry negatively charged phosphate groups were immobilized onto positively charged melamine-modified mica surface via electrostatic attractions, while the negatively charged $d(T)_{20}$ molecules can hardly absorb onto the mica surface. The thickness of the $d(T)_{20}$ layer on the positively charged melamine-modified surfaces was 8.2 nm during the first 1 h, and increased to around 32.8 nm in 2 h. Obviously, the $d(T)_{20}$ molecules formed much more clusters to form a higher surface coverage with the increasing incubation time. The effect of concentration on the thick-

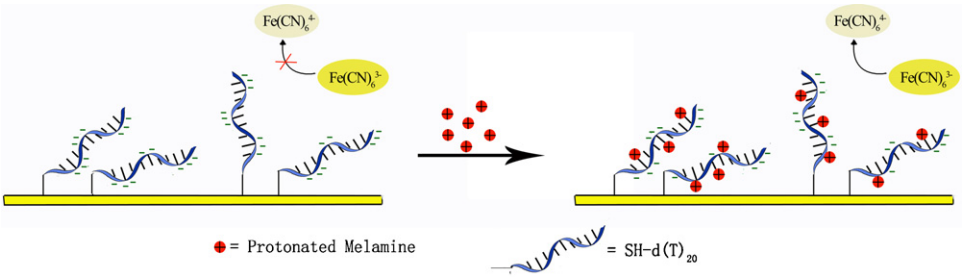

Fig. 2. Schematic representation of the sensing processes.

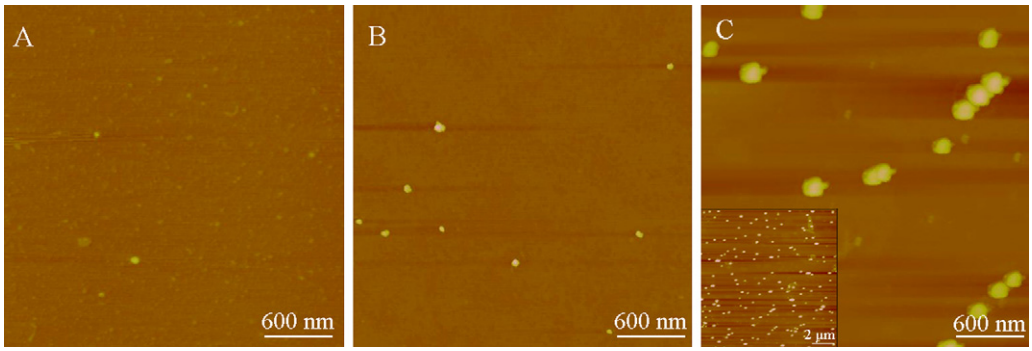

Fig. 3. AFM images (3 μm × 3 μm) of melamine-modified mica surface for different incubation time with $d(T)_{20}$: (A) 0 h, (B) 1 h, (C) 2 h. ($d(T)_{20}$ concentration: 2.2×10^{-5} M). Inset in Fig. 3C shows AFM image (10 μm × 10 μm) of melamine-modified mica surface incubated with 2.2×10^{-5} M $d(T)_{20}$ for 2 h.

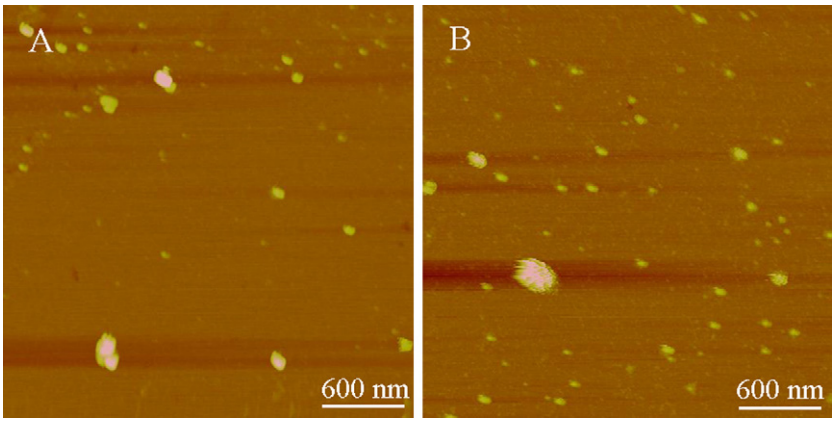

Fig. 4. AFM images (3 μm × 3 μm) of two different surfaces for different concentration of $d(T)_{20}$: (A) 5.4×10^{-6} M, (B) 1.1×10^{-5} M (Incubation time: 4 h).

nesses of the $d(T)_{20}$ overlayers formed on the positively charged melamine-modified surfaces was investigated by applying different concentration of $d(T)_{20}$ solutions (5.4×10^{-6} and 1.1×10^{-5} M, in pH 7.0 phosphate buffer solution) at room temperature. As seen from Fig. 4, more clusters formed on the melamine-modified mica surface with the increasing $d(T)_{20}$ solution concentration since the assembly becomes more convenient at higher concentration of $d(T)_{20}$.

3.2. Optimum conditions for melamine determination

3.2.1. Effect of the amount of $d(T)_{20}$

The electrochemical impedance spectroscopy was used to select a suitable $d(T)_{20}$ concentration for the electrochemical measurements. With increasing $d(T)_{20}$ concentration from 1 to 40 μM, the semicircle diameter in EIS initially increased and then became a plateau at 20 μM or higher. And the large semicircle diameter confirms the high surface density of $d(T)_{20}$ on the gold electrode when the concentration of $d(T)_{20}$ is selected as 20 μM. Therefore, 20 μM of $d(T)_{20}$ was employed for further assay.

3.2.2. Effect of accumulation potential and time

The electrochemical probe of $[Fe(CN)_6]^{3-/4-}$ was used in PBS buffer solution (pH7.0) containing 1.6 μM melamine by DPSV, varying the accumulation potential from 0.2 to 1.0 V, with 30 s accumulation time. The peak current increased with increasing accumulation potential and reached the highest at 0.8 V. Therefore, we select 0.8 V as the optimum accumulation potential.

In the same solution as above, the accumulation time changed from 0 to 300 s when the accumulation potential went to 0.8 V. The peak current value increased slowly with the time, so we selected 30 s as accumulation time to realize the rapid detection.

3.2.3. The pH effect on the melamine–$d(T)_{20}$ interaction

Fig. 5 displays the pH effect on the interaction between $d(T)_{20}$ and melamine. The strength of the interaction between melamine and $d(T)_{20}$/Au electrode is pH dependent. At pH 8.0, melamine is electrically neutral so its electrostatic interaction with the $d(T)_{20}$ is weakened (likely to be due to deprotonation of the amino groups

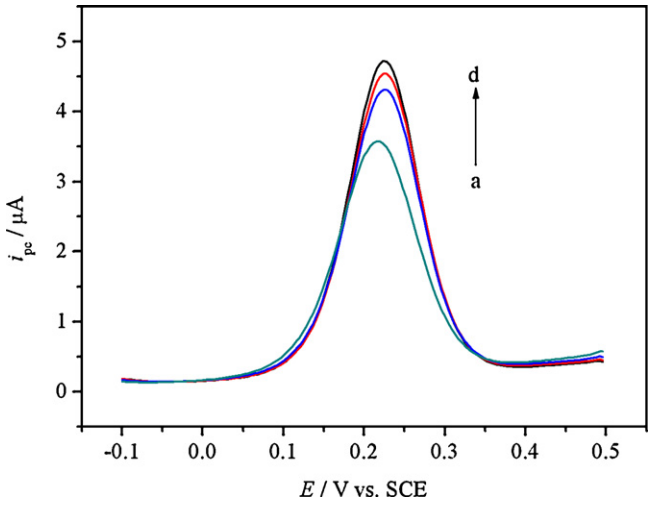

Fig. 5. Differential pulse stripping voltammograms of 1.0 mM $K_3Fe(CN)_6$ mixed with 7.6×10^{-8} M melamine in PBS buffer of different pH values (a–d: the pH values are 8.0, 7.0, 6.0, 5.0) at $d(T)_{20}$/Au.

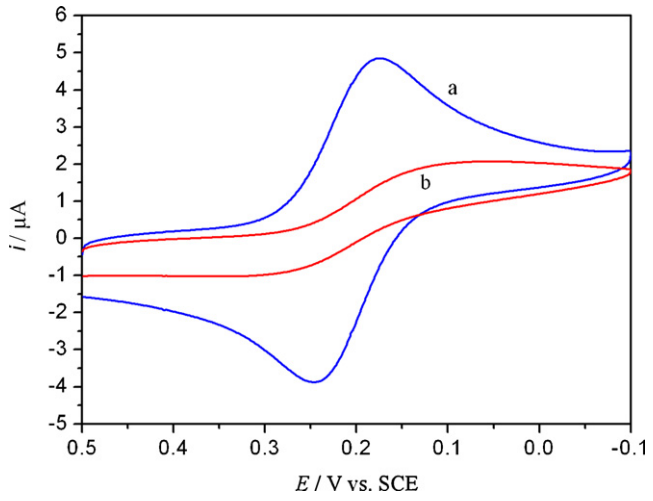

Fig. 6. Cyclic voltammograms of 1.0 mM $K_3Fe(CN)_6$ in pH 7.0 PBS buffer at the bare gold (a) and $d(T)_{20}$/Au (b) with a scan rate of 0.05 V/s.

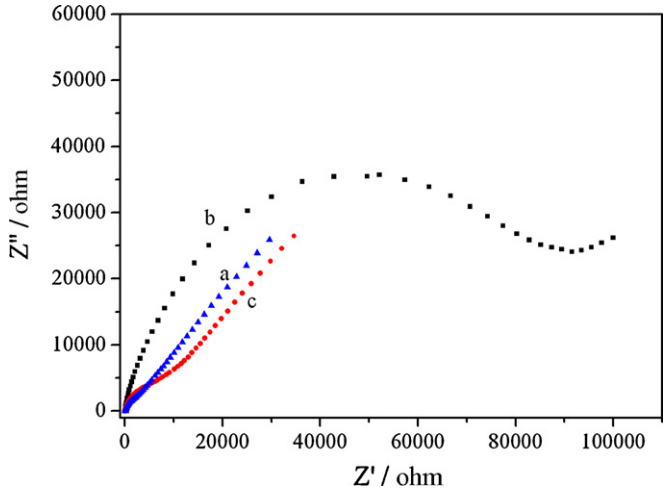

Fig. 7. AC impedance plots of 1.0 mM $K_3Fe(CN)_6$ in pH 7.0 PBS buffer at the bare gold (a), $d(T)_{20}$/Au (b) and $d(T)_{20}$/Au dealed with 6.7×10^{-7} M melamine by DPSV(c).

in melamine). With the decreasing of pH value the peak currents increased, especially from pH 8.0 to pH 7.0. Thus, it is indicated that the interaction becomes stronger in low pH condition, and we presume that the electrostatic interaction between $d(T)_{20}$ and melamine is predominate. To be close to the physiological condition (pH 7.4), we selected pH 7.0 for experiments.

3.3. Electrochemical characteristics of the $d(T)_{20}$/Au

3.3.1. Cyclic voltammograms

The electrochemical behavior of the modified electrode was characterized by cyclic voltammetry using $[Fe(CN)_6]^{3-/4-}$ as an electroactive probe. As shown in Fig. 6, a pair of reversible redox peaks was observed on the bare gold electrode. Compared with the bare gold electrode, the anodic peak potential (E_{pa}) on the $d(T)_{20}$ modified gold electrode shifted from 0.25 to 0.32 V, and the cathodic peak potential (E_{pc}) from 0.17 to 0.06 V, demonstrating that the potential separation between anodic and cathodic peaks ($\Delta E_p = E_{pa} - E_{pc}$) was larger than that of on the bare gold electrode. And the amperometric response of the modified electrode decreased. These results showed that the electron transfer kinetics of $[Fe(CN)_6]^{3-/4-}$ redox reactions was perturbed when the bare gold electrode was modified with the $d(T)_{20}$, confirming that the $d(T)_{20}$ was successfully assembled on gold surface through the Au-S bond.

3.3.2. Impedance analysis

Fig. 7 shows the electrochemical impedance spectroscopy (EIS) at the bare gold electrode and the electrode modified with $d(T)_{20}$. The impedance change of the electrode surface can characterize the stepwise-assemble process of the surface-modified electrode. The typical impedance spectrum (presented in the form of the Nyquist plot) includes a semicircle portion at higher frequencies corresponding to the electron-transfer-limited process and a linear part at lower frequency range representing the diffusion-limited process. The semicircle diameter in the impedance spectrum equals the electron-transfer resistance, R_{et}, which is related to the electron-transfer kinetics of the redox probe at the electrode surface. The response of electrochemical impedance spectroscopy at the bare gold electrode is a straight line (Fig. 7a), revealing there is almost no heterogeneous charge-transfer resistance on bare gold electrode surface, while the impedance response at the modified electrode (Fig. 7b) is much larger, illustrating the immobilization of $d(T)_{20}$ on the electrode. Due to the non-conductivity of $d(T)_{20}$, the introduction of $d(T)_{20}$ layer forms a barrier for electron transfer between the redox probe in the electrolyte solution and the electrode. On the other hand, the negatively charged phosphate groups on $d(T)_{20}$ can also resist the access of the $[Fe(CN)_6]^{3-/4-}$ redox couple to the electrode surface. These results are in good agreement with the results of cyclic voltammetry in Fig. 6.

3.4. The electrochemical determination of melamine

The electrochemical probe of $[Fe(CN)_6]^{3-/4-}$ was used to investigate the interaction between $d(T)_{20}$ and melamine by DPSV. Fig. 8A shows the reductive peak current value of $[Fe(CN)_6]^{3-/4-}$ at $d(T)_{20}$/Au increased with the increasing concentration of melamine in the range from 3.9×10^{-8} to 3.3×10^{-6} M. Fig. 8B shows the linear relationship between the reductive peak current and the concentration of melamine from 3.9×10^{-8} to 3.3×10^{-6} M with a coefficiency of 0.990. The detection limit is up to 9.6×10^{-9} M. These results are attributed to the interaction between melamine and $d(T)_{20}$ on the electrode surface. As we know, the pK_a of melamine is 8.0 [16], hence, the amino groups of melamine could be protonated in pH7.0 PBS solution. Thus, protonated melamine interacted with the negatively charged phosphates of the $d(T)_{20}$ through electrostatic attraction. This suggests that the presence of melamine gives rise to partial charge screening (neutralization) of the negative charges of the phosphate groups of $d(T)_{20}$, which facilitates the electrochemical reaction of $[Fe(CN)_6]^{3-/4-}$ at the $d(T)_{20}$/Au electrode. Meanwhile, melamine can bind to the thymine base through its complementary triamino triazine unit by hydrogen-bonding [17] and specifically combine to the $d(T)_{20}$. Therefore, we presume that the interactions between melamine and $d(T)_{20}$ including electrostatic interaction and hydrogen-bonding. The results of electrochemical impedance spectroscopy can further prove the interaction of $d(T)_{20}$ and melamine (Fig. 7). The radius of the semicircle of $d(T)_{20}$/Au after the interaction with melamine became smaller obviously than that of before the interaction, but it was still lager than that of bare Au. The EIS results indicated that melamine can absorb on the electrode modified with $d(T)_{20}$ and reduce the electron-transfer resistance.

3.5. The selectivity for melamine detection

The influences of some foreign substances on the determination of 9.6×10^{-7} M melamine were investigated. The proposed method showed a good selectivity for melamine detection. For example, 100 fold K^+, Na^+, NH_4^+, Cl^-, PO_4^{3-}, SO_4^{2-}, Ac^-, glucose, alanine, arginine, tyrosine, and monosodium glutamate barely interfere in the determination of melamine.

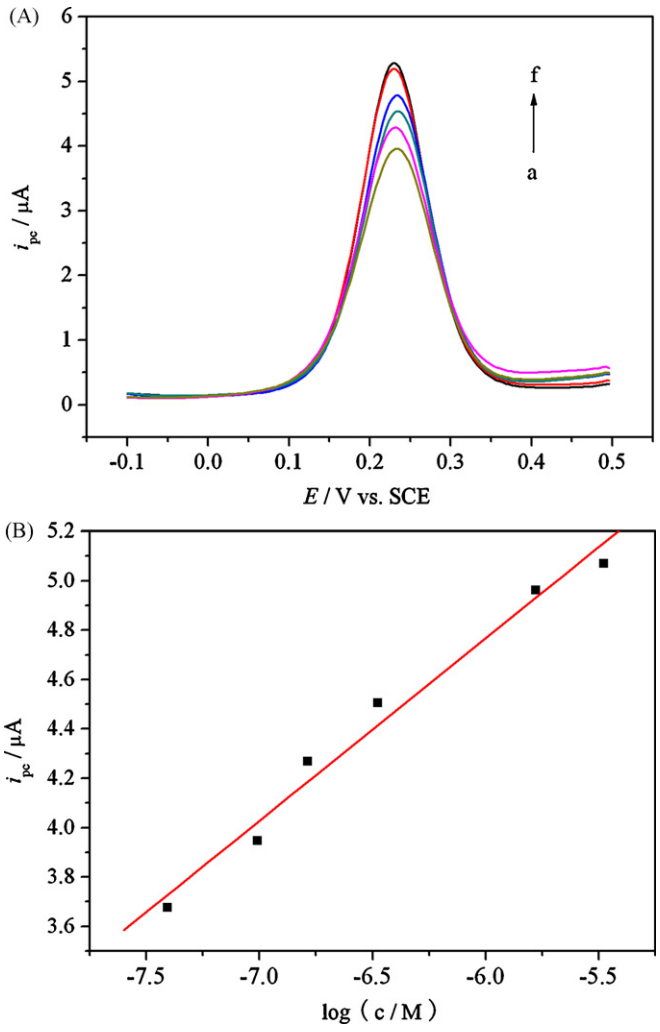

Fig. 8. (A) Differential pulse stripping voltammograms of 1.0 mM $K_3Fe(CN)_6$ in the mixture of melamine in pH 7.0 PBS buffer at $d(T)_{20}$/Au (a–f: the concentration of melamine is 3.9×10^{-8} M, 9.8×10^{-8} M, 1.6×10^{-7} M, 3.3×10^{-7} M, 1.7×10^{-6} M, 3.3×10^{-6} M). (B) Linear relationship between the reductive peak current and the concentration of melamine.

3.6. Stability and reproducibility of $d(T)_{20}$/Au

The stability of the $d(T)_{20}$/Au was examined. After keeping it in refrigerator for two weeks, the current response of the electrode did not remarkably change, showing that the $d(T)_{20}$/Au has good stability.

The reproducibility of the current response for the $d(T)_{20}$/Au was examined in 1.0 mM $K_3Fe(CN)_6$ solution containing 4.8×10^{-7} M melamine. The relative standard deviation was 1.9% for 10 independent determinations.

3.7. Analytical application in milk

To assess its applicability, the proposed method was applied to the analysis of melamine in liquid milk bought from supermarket. The liquid milk was pretreated according to the general procedure. Briefly, 5 g milk was first mixed with 5 mL of 61 mM trichloroacetic acid and 35 mL of 4.9 M methanol solution. After 15 min sonication and 10 min shaking, the mixture was centrifuged at 10000 rpm/min for 10 min, and then the supernatant was filtrated. Next, the filtrate was condensed to give a total volume of 5 mL and filtered through a 0.45 μm filter membrane to obtain the samples for detection. Because the existing milk in the market is free of melamine, the sample was spiked with certain amounts of melamine standard solution directly. The concentration of melamine was calculated to be 3.8×10^{-7} M by standard addition method. The recovery rate was calculated to be 95%, demonstrating the accuracy of the proposed method.

4. Conclusions

In conclusion, we have developed a rapid, simple and reliable method to study the interaction between $d(T)_{20}$ and melamine by using $K_3Fe(CN)_6$ as an electrochemical indicator. Melamine might interact with $d(T)_{20}$ in two modes, electrostatic attraction and hydrogen-bonding. The proposed electrochemical sensor has a good sensitivity for the detection of melamine, and the peak current of $K_3Fe(CN)_6$ linearly increased with the increasing concentration of melamine over a range from 3.9×10^{-8} to 3.3×10^{-6} M. The detection limit of the proposed sensor is lower than that of traditional methods. The proposed method was applied successfully to the determination of melamine in milk products, and the recovery was 95%. Thus this sensor is expected to have a potential application in food inspection.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Grant Nos. 20571085 and 20877099), 973 Project (Grant No. 2007CB935900), the State Key Laboratory of Natural and Biomimetic Drugs (Grant No. 20080208), President Fund of GUCAS (Grant No. 065101HM03) and (water special project (Grant No. 2008ZX07527-007)).

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