Microchim Acta (2015) 182:2069–2075
DOI 10.1007/s00604-014-1414-3

SHORT COMMUNICATION

Electrochemical determination of pyrophosphate at nanomolar levels using a gold electrode covered with a cysteine nanofilm and based on competitive coordination of Cu(II) ion to cysteine and pyrophosphate

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Received: 29 August 2014 / Accepted: 11 November 2014 / Published online: 14 January 2015

Abstract We are presenting an electrochemical method for the determination of pyrophosphate ions (PPi) that is based on the competitive coordination of Cu(II) ion to a nanofilm of cysteine (Cys) and dissolved PPi. Cys was immobilized on the surface of a gold electrode by self-assembly. The Cys-modified gold electrode was loaded with Cu(II) ion which is released from the surface on addition of a sample containing PPi. The sensor shows an unprecedented electrochemical response to PPi, and the reduction peak currents is linearly related to the logarithm of the concentration of PPi in the 100 nM to 10 mM range (with an R^2 or 0.982). The limit of detection is ~10 nM which is lower than the detection limits hitherto reported for PPi. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and common anions give a much weaker response. The method demonstrated here is simple, effective, highly sensitive, hardly interfered, and does not require the addition of a reagent. The method was applied to the determination of PPi in (spiked) serum samples.

Keywords Pyrophosphate ion sensing · Copper ion · Competitive coordination · Cysteine

Introduction

Pyrophosphate (PPi) is one of the by-products of adenosine triphosphate (ATP) hydrolysis to produce energy; it also participates in bioenergetic processes such as ATP hydrolysis. In mammals, PPi plays an essential role in calcification processes [1, 2] and in cancer diagnosis by monitoring telomerase elongation process [3]. Detection and quantification of PPi can also help identify diseases such as chondrocalcinosis or calciumpyrophosphate crystal deposition disease [4, 5]. Considering its importance, great efforts have been made to establish analytical methods for the selective detection and recognition of PPi.

Though enzymatic methods for PPi assay are very sensitive and convenient, they need specific enzymes, substrates as well as special equipment and unfortunately narrow their applications [6]. Therefore, the design of selective and sensitive chemosensors for PPi based on absorption [7], color [8, 9], chemiluminescence [10] and fluorescence [11–13] changes has been very popular during the past 10 years. Fluorescent probes have been widely designed for recognizing or detecting phosphate-containing anions [14, 15], in which metal ions with a variety of organic ligands are generally designed as the binding site for PPi with the aid of strong binding between the metal ions and PPi. Owing to the coordination of PPi with Zn^{2+}, bis(2-pyridylmethyl)amine Zn^{2+}, dipicolylamine(dpa)-Zn(II) complex and terpyridine-Zn(II) complex strategies were designed as efficient and highly selective fluorescent probes for PPi [16–19]. However, certain key issues still remain to be addressed. For instance, the selectivity of all these organic artificial probes for PPi against other phosphate-containing anions such as ATP, guanosine triphosphate (GTP) must be considered during the design [20, 21]. In addition, due to strong hydration of anions and low solubility of organic fluorescent probes, most are limited to organic or mixed aqueous media [22, 23], which limited their biological applications. Although a few recent studies have addressed this issue, the sensitivity in water (or in organic

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or mixed media) was invariably limited to micromolar levels [15, 24, 25]. Therefore, there is a growing demand for highly selective, sensitive and effective technically simple probes for PPi detection for diagnostics and medical applications.

It was reported L-cysteine molecules contain amino group (-NH_2) and carboxyl (-COOH), chelating copper (II) ions (Cu^{2+}) with a five membered ring [26]. Moreover, the stability constant (K) of the complex formed by Cu^{2+}andcysteine is $\log K_{Cu\ Cysteine}$ =7.88 [27], and that for the complex formed by $Cu^{2+}$and PPi is $\log K_{Cu\ PPi}$ =12.45 [28]. The complexation constants suggested coordination effect between PPi and copper ion is stronger than between cysteine and copper ion. Based on this mechanism, Deng et al. demonstrated two colorimetric methods for selective PPi sensing and pyrophosphatase activity assay with Au nanoparticles (Au-NPs) as the signal readout [29]. Li etc. reported that Cys-modified gold electrode can load Cu(II) ion and the captured Cu^{2+} behaviors excellent electrochemical properties [30]. Accordingly, other than these colorimetric methods, competitive coordination chemistry of Cu^{2+} between cysteine and PPi essentially could form a straightforward basis for PPi electrochemical sensing with Cu^{2+}/Cys/Au electrode as readout signal. As far as we know, there is still lack of electrochemical sensing method for PPi detection based on electrochemical signal of Cu^{2+} inspired by this competitive coordination reactivity of copper ions (Cu^{2+}) between cysteine and PPi.

We developed an effective electrochemical method for nanomolar level detection of PPi in water at physiological pH utilizing a simple competitive coordination reactivity of Cu^{2+} between cysteine and PPi on the Cu^{2+}/Cys/Au electrode. Upon the presence of PPi, a four membered ring with Cu^{2+} was formed and Cu^{2+} were disassociated from Cu^{2+}/Cys/Au electrode, leading to the current signals from the Cu^{2+} reduction decrease and enable to detect the PPi (Scheme 1). The electrochemical sensor has an improved detection limit as compared to that of the existing PPi sensors and exhibits excellent PPi sensing selectivity over other anions and structurally analogues. To the best of our knowledge, the study actually paves an electrochemical route to the fast and simple clinic detection of PPi.

Experimental

Materials

L-cysteine, $K_3[Fe(CN)_6]$, KH_2PO_4, acetic acid, sodium acetate, ATP, adenosine monophosphate (AMP), adenosine diphosphate (ADP) purchased from Beijing Chemical Reagent Company (www.crc-bj.com)., $NaNO_3$, Na_2SO_4, KCl, Na_3PO_4, Na_2HPO_4, KH_2PO_4, Na_2CO_3, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich (Shanghai, China) (www.sigmaaldrich.com). And all aqueous solutions were prepared with double-distilled water produced by a Milli-Q system (Millipore, Bedford, MA, USA, 18.2 MΩ.cm, www.merckmillipore.com).

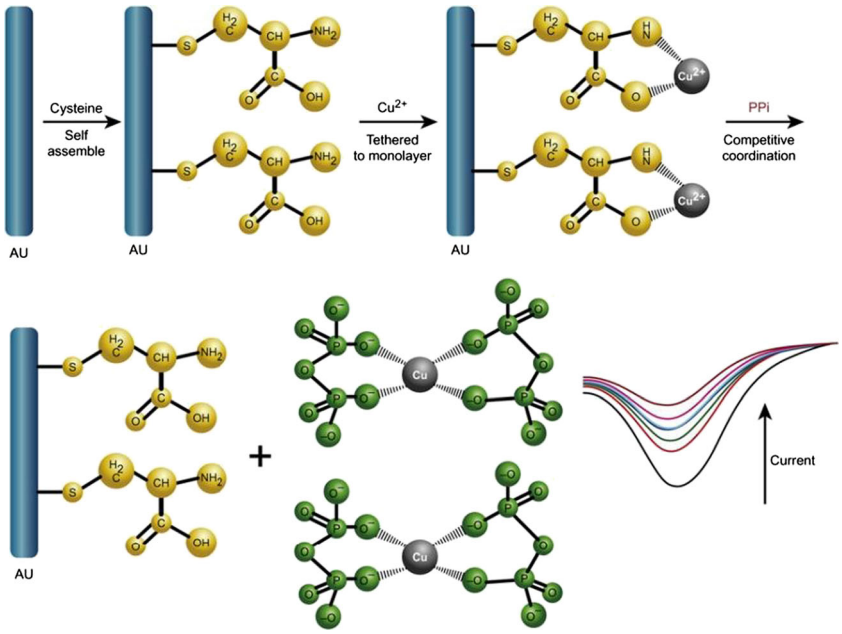
Instrumentation

Electrochemical measurements were carried out with a computer-controlled electrochemical analyzer (CHI 600E, Chenhua, China) and a computer-controlled advanced electrochemical system (Princeton Applied Research (PARST AT 2273) in a two-compartment electrochemical cell with a bare or modified Au electrode (2 mm in diameter) as working electrode, a platinum wire as counter electrode, and an Ag/AgCl electrode (KCl-saturated) as reference electrode. Au electrodes (0.18-cm diameter, Bioanalytical Systems, Inc.) were pretreated as described previously [31, 32].

Electrochemical measurements

Au electrodes modified with self assembled monolayer (SAM) of cysteine, i.e. Cys/Au electrode, were prepared by immersing the electrodes into 0.10 M acetate buffer (pH 5.0) containing 8.0 mM cysteine for 30 min. The cysteine-modified Au electrodes (Cys/Au) were then thoroughly rinsed with doubly distilled water and finally dried with pure N_2. The electrodes were then run cyclic voltammetry (CV) in 0.10 M acetate buffer (pH 5.0) solution to get a stable CV curves. Preconcentration of Cu^{2+} was accomplished by immersing the Cys/Au electrode into 0.10 M acetate buffer (pH 5.0) containing Cu^{2+} for 10 min under magnetic stirring to form Cu^{2+}/Cys/Au electrode. The electrodes were then thoroughly rinsed with doubly distilled water to remove Cu^{2+} unstably adsorbed onto electrode surface and the voltammograms were recorded in Cu^{2+}-free 0.10 M acetate buffer (pH 5.0).

To investigate the detection of PPi, initially, the Cu^{2+}/Cys/Au electrode was immersed at different concentrations of PPi solution with stirring 30 min,then Cu^{2+}/Cys/Au electrode was stirred 10 min at PPi-free 10 mM HEPES buffer (pH 7.4) and then CVs was recorded. In attempt to confirm the selectivety of this study,different kinds of species and interference ions including ATP, AMP, ADP, $NaNO_3$, Na_2SO_4, KCl, Na_3PO_4, Na_2HPO_4, KH_2PO_4, Na_2CO_3 were added into 10 mM HEPES buffer (pH 7.4) solution separately. After interact with Cu^{2+}/Cys/Au electrode using the same procedure as detection of PPi, CVs were recorded.



Scheme 1 Schematic illustration of the PPi sensing process

Serum preparation

Collect whole blood in a covered test tube and allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 15–30 min. Remove the clot by centrifuging at 1000–2000×*g* for 10 min in a refrigerated centrifuge. The resulting supernatant is designated serum. The samples should be maintained at 2–8 °C while handling. If the serum is not analyzed immediately, the serum are stored, and transported at −20 °C or lower.

Statistical analysis

For the statistical analysis of the PPi levels in the diluted serum samples spiked with different concentrations of PPi, the original current responses recorded with the Cu^{2+}/Cys/Au electrode were converted into the concentration (μM) of PPi according to the linear equation described. The data were presented as the mean±standard deviation (SD). Statistical significance was determined by two-tailed Student's *t*-test with 95 % confidence for unpaired observations. A *p* value less than 0.05 was considered to be significant.

Results and discussion

Electrochemical characteristics and optimization of experimental condition

To enable the electrochemical methods effective for reliable determination of Cu^{2+}left on the surface of electrode after interaction with the PPi, we report that SAM of cysteine was formed onto Au electrodes since cysteine could well attach to the Au surface both via the strong S-Au bond preventing the

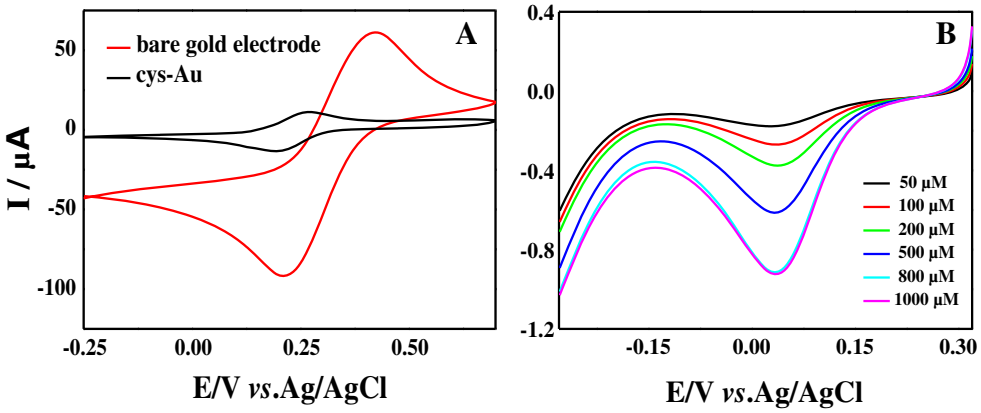


Fig. 1 **a** CVs of Cys/Au electrode and bare Au electrode in 1.0 mM $K_3[Fe(CN)_6]$ solution. **b** The reduction parts of CVs of Cu^{2+}/Cys/Au electrode after interact with different concentrations Cu^{2+}. Scan rate: 10 mV · s^{-1}

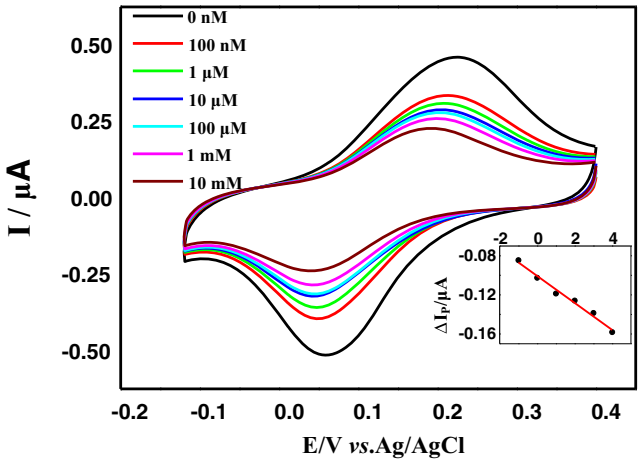
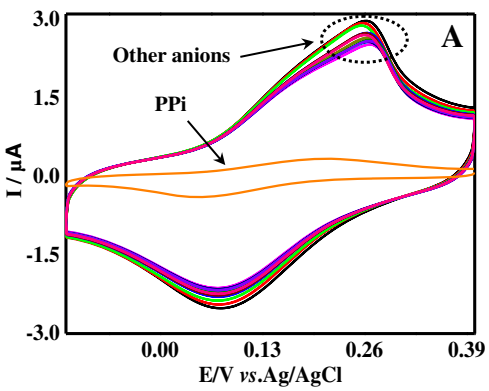


Fig. 2 CVs of Cu^{2+}/Cys/Au electrode after interact with different concentrations of PPi in HEPES buffer solutions (10 mM, pH 7.4). Scan rate: 10 mV · s^{-1}. Inset: the linear relationship between ΔI_P and the logarithm of Cu^{2+} concentration within a concentration range from 100 nM to 10 mM. Cu^{2+}/Cys/Au electrodes were obtained by preconcentrating Cys/Au electrodes in 500 μM Cu^{2+}

nonspecific adsorption of some big molecule like peptide and protein in biological sample. Moreover, coordination of Cu^{2+} with the acidic (−COOH) and basic ($-NH_2$) functional groups of cysteine under weakly acidic conditions eventually validates the detection of low concentration of $Cu^{2+}$and thus for indirect sensing of PPi.

Figure 1a shows CVs of a bare Au electrode and a Cys/Au electrode recorded in the1.0 mM $K_3[Fe(CN)_6]$ solution. The pair of redox peaks for bare electrode was remarkable larger than that for the Cys/Au electrode, which suggested that self-assembled monolayers of cysteine was successfully assembled onto Au electrode. This phenomenon is ascribed to that the surface of Au electrode modified by cysteine molecule monolayers prevents electron transfer, i.e. redox reaction on the surface of Au electrode.

Successful preconcentration of Cu^{2+} on the Cys/Au surface was evident from the presence of well defined anodic (+ 0.27 V) and cathodic (+0.12 V) peaks at the electrode after the electrode was treated with 0.10 M acetate buffer (pH 5.0) containing Cu^{2+} and then taken out from the solution and washed with water (data not shown). These peaks were attributed to the redox process of Cu^{2+} tethered onto electrode surface. The peak currents for Cu^{2+} remain very stable, almost showing no decrease after consecutively potential cycling for at least 50 cycles. These properties substantially enable the electrochemical method effective for investigation of PPi by monitoring Cu^{2+} remaining on the cysteine modified electrodes. To choose the proper concentration of Cu^{2+} for tether Cu^{2+}on the Cys/Au electrode, Cu^{2+}preconcentration with different concentration on the Cys/Au electrode was investigated. As can be seen from Fig. 1b, the reduction part of CV recorded in acetate buffer (0.1 mol/L, pH5.0) buffer solution increase obviously after the interaction with different concentrations Cu^{2+}. The well-defined current responses of Cu^{2+} at the Cys/Au enable the electrochemical method practically applicable for monitoring Cu^{2+}. On the other hand, no redox current response was observed for both bare and Cys/Au electrode without the treatment of Cu^{2+} (data are not shown). Furthermore, the current of reduction peak of Cu^{2+}/Cys/Au electrode is linearly increased along with the concentration of Cu^{2+} from 50 to 500 μM. When the concentrations of Cu^{2+} ranged from 800 to 1000 μM, the current of reductive peak of Cu^{2+}/Cys/Au electrodes are scarcely increased and stay around maximum, which indicates that active sites of cysteine formed onto Au electrodes have fully been occupied by Cu^{2+}according to the chelation after the concentration of Cu^{2+} increased to 800 μM. In addition, when the Cys/Au electrode was preconcentrated with 500 μM Cu^{2+}, the reductive peak of Cu^{2+}/Cys/Au electrode is more stable with a better peak shape, thus, the concentration



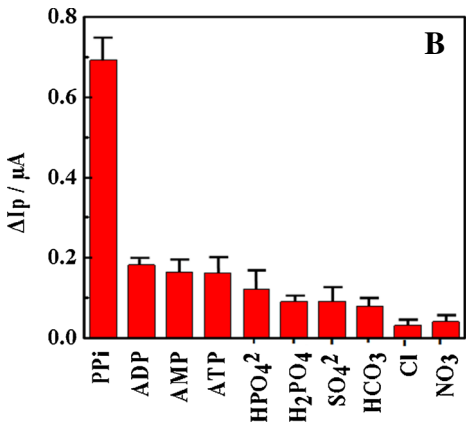


Fig. 3 **a** CVs recorded of Cu^{2+}/Cys/Au electrode after interact with 1 mM PPi as well as various biologically relevant anions including ATP, AMP, ADP, Cl^-, SO_4^{2-}, $H_2PO_4^-$, NO_3^-, HCO_3^- and HPO_4^{2-} in HEPES buffer solutions (10 mM, pH 7.4). Scan rate: 10 mV · s^{-1}. **b** Relative reduction peak currents intensity to different anions according to A. Each value was derived from three independent detections and error bars mean standard deviation (SD)

Table 1 Figures of merit of electrochemical methods for determination of PPi

Method	Linear range	Limit of detection	Comments	Ref
Absorption spectroscopy and conductivity	5–50 μM	0.64 μM	Accurate; high selectivity; reliability; inexpensive.	[7]
Absorption spectroscopy and color	0–400 μM	–	High affinity and selectivity; work at a high electrolyte concentration and in blood plasma.	[8]
Fluorescence	0–100.0 μM	1.0 μM	Selective; the first examples of charge neutral fluorescent Photoinduced Electron Transfer (PET) probe	[11]
Fluorescence	0–12 mM	–	Easily synthesized; sensitive; high selectivity.	[12]
Electrochemical	100 nM to 10 mM	10 nM	Simple; highly sensitive; hardly interfered.	This work

of Cu^{2+}solution was chose as 500 μM to preconcentrate Cu^{2+} and to obtain a Cu^{2+}/Cys/Au electrode for next PPi sensing.

Quantification of PPi

We describe a chelation-based sensor to surface-sensitive electrochemical detection, requiring a chelator, i.e. cysteine compatible with surface immobilization and selective to the target analyte. Surface capture of signaling molecules, i.e. Cu^{2+}, is expected to enhance the sensitivity of electrochemical detection to PPi in a process that we call "signal immobilization". The negatively-charged PPi molecules are expected to decrease the number of Cu^{2+} on the surface of the electrode functionalized with cysteine as a chelator, resulting in a decrease in reduction currents. In this "signal immobilization" concept, cysteine act as a chelator with three functional components: a binding site, a linker, and a handle. The binding site captures and preconcentrates Cu^{2+} from solution, while the linker between the binding site and handle provides steric flexibility. Finally, the handle –SH group ensures that the chelator can be attached to a chemically compatible surface, i.e. Au electrode.

To investigate the different concentration of PPi sensing with Cu^{2+}/Cys/Au electrode, CVs of Cu^{2+}/Cys/Au electrode with different concentrations of PPi in HEPES buffer solutions (10 mM, pH 7.4) was recorded (Fig. 2). From the Fig. 2, Cu^{2+}/Cys/Au electrode was immersed into different concentration of PPi solution stirred 30 min, the peak current of Cu^{2+}/Cys/Au electrode become more and more smaller with the concentration of PPi increase. This can be attributed to that PPi has more strong coordination effect with Cu^{2+} than cysteine with Cu^{2+} inducing Cu^{2+}dissociated from the Cu^{2+}/Cys/Au electrode and thus the peak currents decrease, as demonstrated in Scheme 1. Besides, inset of Fig. 2 shows that the changes in the value of reduction currents, ΔIp, recorded from Cu^{2+}/Cys/Au electrodes before and after interacting with different concentration of PPi were linear with the logarithm of PPi concentration within a concentration range from 100 nM to 10 mM ($\Delta Ip/\mu A = -0.1006–0.0138 \cdot lgcPPi/\ \mu M$, $R^2 = 0.982$, the standard error for intercept and slope are 0.0019 and 0.00084, respectively) with the detection limit of 10 nM, which fit well for detection PPi in some biological fluids such as plasma and serum in which PPi was found to be around 2.14–3.30 and 4.62–7.51 μM, separately [33]. The sensitivity was higher than other fluorescent probes and Au-NP-based colorimetric assays [16–19, 34]. The low detection limit may be due to the excellent electrochemical signals of Cu^{2+}/Cys/Au electrode and obviously comparable stability constant of the complex formed by Cu^{2+} and cysteine and Cu^{2+} and PPi. In addition, this assay is much easier and more robust than the enzyme-coupling pyrophosphate methods that require at least two enzymes for their pyrophosphate detections.

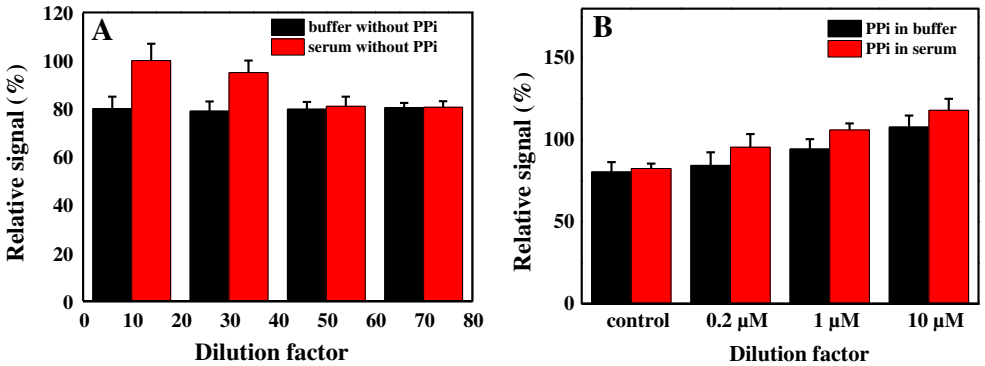


Fig. 4 Results obtained from the testing of (**a**) HEPES buffer solutions (10 mM, pH 7.4) solution and serum samples with different dilution ratios without PPi, and (**b**) 50-fold diluted serum samples spiked with different concentrations of PPi. Each value was derived from five independent detections and error bars mean SD

Detection specificity

To investigate the selectivity of our method, different kinds of species that were considered to possibly interfere with the PPi sensing were interacted Cu^{2+}/Cys/Au electrode. In Fig. 3a, CVs were recorded after 1 mM of various biologically relevant anions including ATP, AMP, ADP, Cl^-, SO_4^{2-}, $H_2PO_4^-$, NO_3^-, HCO_3^- and HPO_4^{2-} were interact with Cu^{2+}/Cys/Au electrode, respectively. The currents of redox peaks of Cu^{2+} tethered to the Cys/Au electrode have relatively small changes after Cu^{2+}/Cys/Au electrode after interact with the interference anions. However, with the presence of PPi under identical conditions, the current of redox peak of Cu^{2+}/Cys/Au electrode decreased obviously, which can also be confirmed in Fig. 3b. These results substantially suggest that these anions did not interfere with the PPi sensing and the developed mechanism for PPi sensing is strong anti-interference. In order to compare with other methods for determination of PPi, we summarize the specific features of each method in Table 1, which shows our electrochemical method is comparable with other methods, especially it is a cost-effective, and process-simple method with wide linear range and low limit of detection. These property substantially enables the utilization ofthe competitive coordination of Cu(II) ion to a nanofilm of cysteine (Cys) and dissolved PPi offering a technically simple effective electrochemical approach to indirect sensing of PPi biomarker in biological sample.

Electrochemical detection of PPi in human serum sample

In an attempt to explore the validity of our electrochemical method for PPi sensing low to nanomolar for practical applications, we tested a real human serum sample. First, the tolerance of the present method under the different dilution rates of human serum was investigated by analyzing different dilution ratios (1:10,1:30,1:50, 1:70) of human serum without PPi, as shown in Fig. 4a. It was obvious that the absorbance values from the diluted serum were comparable to those from HEPES buffer solutions when the dilution ratio was up to 1:70. Then, the 50-fold dilution of human serum samples were spiked with PPi three concentrations (200 nM, 1 and10 μM) and measured. As shown in Fig. 4b, comparable responses were found for PPi in both buffer and serum. The results showed that the designed PPi sensor still worked well in real serum samples. As a result, it might be promising for the determination of PPi in clinical test.

Conclusion

In summary, by rationally tailoring the electrode surface through the competitive coordination interaction of Cu^{2+} between cysteine and PPi and exploring the excellent electrochemical property of Cu^{2+}, we have successfully demonstrated a highly sensitive and selective method for electrochemical sensing of PPi. The developed method offered several advantages over current PPi detection techniques. First, the electrochemical method require neither specific enzymes, substrates as well as special complicated and expensive equipment, nor intricate multi-step synthesis processes and application in organic or mixed aqueous media inorganic of fluorescent probes, which can be readily be recorded by a simple electrochemical station. Second, using this Cu^{2+} tethered Cys/Au electrode sensor, the limit of PPi detection was as low as 10 nM, which was useful for rapid and ultrasensitive detection of PPi. Third, the sensor showed excellent specificity to target PPi over other anion competitors. Finally, this method was well validated for PPi determination in human serum samples. These advantages substantially made this method very promising for the reliable sensing of PPi in diagnostics and other clinical applications.

Acknowledgments This work was financially supported by National Natural Science Foundation (21375088), Scientific Research Project of Beijing Educational Committee (KM201410028006), Youth Talent Project of the Beijing Municipal Commission of Education (CIT&TCD201504072), Scientific Research Base Development Program of the Beijing Municipal Commission of Education and the 2013 Program of Scientific Research Foundation for the Returned Overseas Chinese Scholars of Beijing Municipality.

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