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Electrochemical Sensing of Alkaline Phosphatase Activity Based on Difference of Surface Charge of Electrode

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Alkaline phosphatase (ALP) is commonly used in clinical assays as a biomarker for the diagnosis of many diseases. ALP inhibitors have been considered as promising drugs for curing the diseases caused by ALP-overexpression. In this work, we reported an electrochemical strategy for probing of ALP activity and screening of potential ALP inhibitors based on the difference of interfacial features of electrode. Specifically, in the absence of ALP, phosphorylated peptides (Cys-Pro-pTyr-NH_2 denoted as CP_PY) were immobilized onto the gold electrode to form the negatively charged self-assembled monolayers (SAMs), which facilitated the access of the positively charged $[Ru(NH_3)_6]^{3+}$ probes to the electrode surface. As a result, a couple of adsorption post-wave from $[Ru(NH_3)_6]^{3+}$ was observed. When the electrode was incubated with CP_PY and ALP together, the phosphate group in CPpY would be removed by ALP. Consequently, no apparent post-wave was observed on the modified electrode. The magnitude of the decrease in the post-current was related to ALP concentration and its activity. To demonstrate the feasibility of the method for screening the potential ALP inhibitors, levamisole, a well-known ALP inhibitor, was tested. The results indicated that the method is suitable for probing ALP activity and screening ALP inhibitors in laboratory research.

Keywords: Dephosphorylation; Alkaline phosphatase; Activity; Electrochemistry; Inhibitors

1. INTRODUCTION

Phosphorylation and dephosphorylation play pivotal roles in biological functions and controls nearly every cellular process, including metabolism, gene transcription and translation, cell-cycle progression, cytoskeletal rearrangement, protein-protein interactions, protein stability, cell movement, and apoptosis [1]. A simple and sensitive assay to report the change of phosphorylation state is extremely valuable for biomedical applications. Phosphatase is an enzyme that can remove a

phosphate group from the substrate by hydrolysing phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group. The process of removing the phosphate group is called dephosphorylation. Among kinds of phosphatases, alkaline phosphatase (ALP, EC 3.1.3.1) is the common phosphatase in many organisms [2]. For example, in humans, ALP is present in all tissues throughout the entire body. In clinical assays, ALP is considered as a biomarker for the diagnosis of many diseases, including bone diseases, liver dysfunction, prostatic cancer and bile duct blockage [3]. ALP inhibitors have also been shown to be promising drugs for curing the diseases caused by ALP-overexpression [4]. Moreover, ALP has become a useful tool in molecular biology laboratories as an enzymatic label [5]. Therefore, simple and sensitive method for probing of ALP activity and screening of potential ALP inhibitors has important clinical implications.

To date, there have been some methods for probing of the change of phosphorylation state and the detection of ALP and its activity, such as fluorescence spectrophotometry, lectin method, chemiluminescence analysis, surface enhanced Raman spectroscopy, electrochemistry, colorimetric assay and so on [6-16] Among these methods, electrochemical techniques are simple, sensitive, and inexpensive to implement for these investigations. For example, Kraatz's group reported the electrochemical detection of kinase-catalyzed phosphorylations of the surface-bound peptides using 5′-γ-ferrocenyl (Fc)-adenosine triphosphate (Fc-ATP) [15]. Kerman et al. reported the electrochemical detection of protein phosphorylation based on the labeling of a specific phosphorylation event with gold nanoparticles [13]. In this work, the phosphorylation reaction is coupled with the biotinylation of the kinase substrate using a biotin-modified adenosine 5'-triphosphate [γ]-biotinyl-3,6,9-trioxaundecanediamine (ATP) as the co-substrate. Miao et al. developed a novel electrochemical biosensor for the detection of ALP by the use of two complementary DNA probes coupled with λ-exonuclease [11]. Very recently, Ino et al. reported an electrochemical detection system for ALP activity based on the difference in water and oil solubilities between ferrocene ethyl phosphate ester ($FcEtOPO_3^{2-}$) and ferroceneethanol (FcEtOH, the enzymatic product of $FcEtOPO_3^{2-}$) [16]. However, these amazing methods need the use of expensive bioreagents, labeled target or nanoparticles for signal amplification, which increases the operation complexity and assay cost.

Self-assembled monolayers (SAMs) provide a convenient, flexible, and simple system with which to tailor the interfacial properties of metals, metal oxides, and semiconductors [17]. The terminal groups of SAMs have a great impact on the redox response and electron-transfer resistance of redox probes in aqueous solutions due to the electrostatic interaction between the terminal groups and ionic redox species. For example, the voltammetric response of $[Fe(CN)_6]^{3-/4-}$ at the SAM-modified electrode is decreased in the order of the terminal group NH_2 > OH > COOH, while the response of $[Ru(NH_3)_6]^{3+/2+}$ is increased in the order of NH_2 < OH < COOH [18,19]. Based on these properties, the electrostatic binding of redox-active metal cations (e.g., $[Ru(NH_3)_6]^{3+}$) to negatively charged DNA has been widely used to detect nucleic acids, proteins and small molecules [20-27]. Recently, our group investigated the carbodiimide-mediated amine coupling reaction based on measurements of the electrochemical response of SAMs in the presence of $[Fe(CN)_6]^{3-/4-}$ as a redox probe [18]. The major difference in the process of dephosphorylation is the negatively charged phosphate group. In the present work, we reported a simple and label-free electrochemical method for probing of ALP activity and screening of ALP inhibitors based on the difference of interfacial features of the electrode.

2. EXPERIMENTAL

2.1 Chemicals and materials

The amidated tripeptide (Cys-Pro-Tyr-NH_2, CPY) and its phosphorylated form (Cys-Pro-pTyr-NH_2, CP_PY) were synthesized and purified by ChinaPeptides Co., Ltd (Shanghai, China). 6-Mercapto-1-hexanol (MCH), $[Ru(NH_3)_6]Cl_3$, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), thrombin and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were obtained from Sigma-Aldrich. Alkaline phosphatase (ALP) and levamisole were obtained from Sangon Biotech. Co., Ltd. (Shanghai, China). The $[Ru(NH_3)_6]^{3+}$ solution was prepared with 50 mM HEPES buffer (pH 7.0). The ALP solution was prepared with deionized water, and diluted to the desired concentration before use. The deionzied water was treated with a Millipore system (Simplicity Plus, Millipore Corp.).

2.2 Detection of ALP activity

The gold disk electrode with a diameter of 2 mm was polished with diamond pastes down to 3 mm and alumina pastes down to 0.3 mm, and then sonicated in ethanol and water. CP_PY (20 μM) was allowed to react with different concentrations of ALP in pH 8.0 PBS solution for 1 h. The cleaned gold electrodes were then immersed into the mixed solution containing 50 μM TCEP in the darkness overnight at room temperature. This step was followed by washing the electrode thoroughly with water and soaking the electrode in a 1 mM MCH solution for 1 hour to block the unreacted gold surface. Again, the electrode was rinsed with ethanol/water to rid any non-specifically adsorbed substance. The experimental conditions for the formation of SAMs on gold electrode were chosen according to the well-established protocol [28]. Voltammetric determination in N_2-saturated $[Ru(NH_3)_6]^{3+}$ solution was performed on a uECS-PRO electrochemical workstation (Changchun Institute of Applied Chemistry Chinese Academy of Science, China) using a homemade plastic electrochemical cell. For mass spectrometric analysis, peptide CP_PY was incubated with pH 8.0 PBS solution containing ALP for 1 h at room temperature, following by the addition of 0.1% TFA. Electrospray ionization/time of flight mass spectrometry (ESI-TOF-MS) was conducted on a LCT Premier XE mass spectrometer.

3. RESULTS AND DISCUSSION

3.1 Principle of the method

The analysis principle of the present work is shown in Fig. 1. It is based on measurements of the electrochemical response of CP_PY/MCH-covered gold electrodes in the presence of the positively charged $[Ru(NH_3)_6]^{3+}$ as a redox probe. Because the negatively charged CP_PY/MCH SAMs facilitate the access of the redox probe to the electrode surface (Path I), the diffusion- and adsorption-controlled waves will be observed in the $[Ru(NH_3)_6]^{3+}$ solution. If the phosphate groups of CP_PY are removed by ALP, the neutral CPY/MCH SAMs will reduce the amount of absorbed $[Ru(NH_3)_6]^{3+}$ and the adsorption post-current (Path II). The magnitude of the decrease in the peak current is related to the amount of phosphate groups on electrode, which depends upon the concentration and activity of ALP.

Note that we also attempted to detect ALP directly using CP_PY/MCH-covered electrode (Path III). As a result, no obvious current change was observed at the modified electrode in $[Ru(NH_3)_6]^{3+}$ solution before and after incubation with ALP solution for 6 h. It is worth nothing that we conducted the mass spectrometry of the mixture of CPpY and ALP, and found that the phosphate group in the synthesized CPpY peptide can be removed by ALP in solution within 1 h (Fig. 2). Therefore, we suggested that the immobilized CP_PY molecules on electrode (Path III) are unfavorable to react with ALP due to the effect of steric hindrance, and started the ALP detection by incubating ALP with CP_PY before the self-assembly.

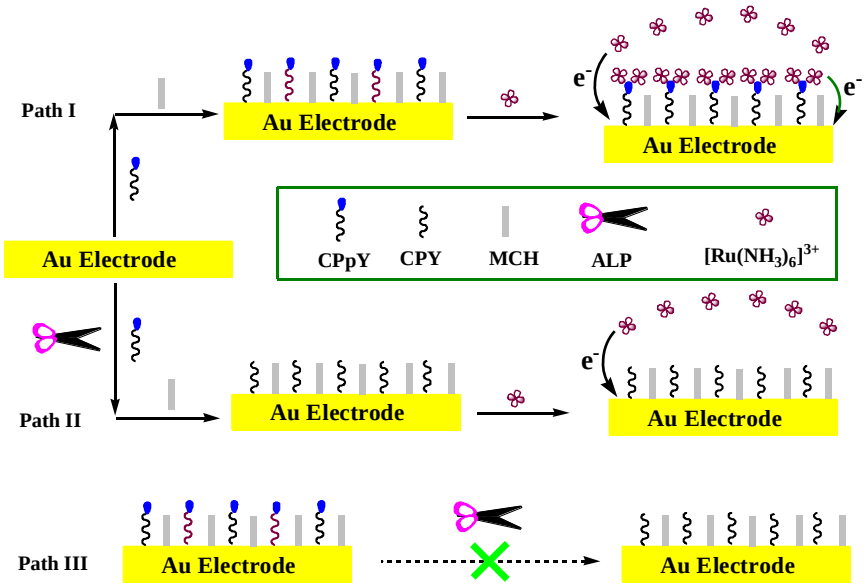

Figure 1. Scheme representation showing the strategy for probing of ALP activity based on the difference of interfacial features of the electrode.

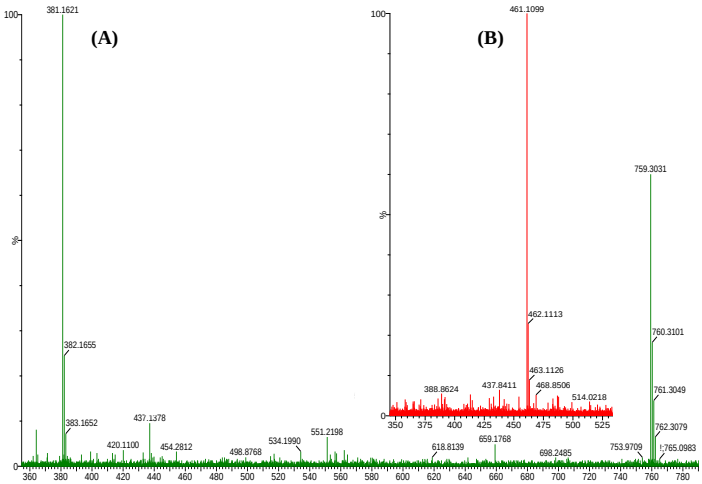

Figure 2. ESI-TOF-MS spectrum of 20 μM CPpY with (A) and without (B) incubation with 200 ng/mL ALP. The dominant mass peak at 381.1621 Da in panel A (green) belongs to CPY, the reaction product of CPpY after stripping the phosphate group. The mass peak at 759.3031 Da is assigned to the oxidation production of CPY due to the formation of disulfide in air-saturated solution. Therefore, TCEP was added into the reaction mixture before the self-assembly. The dominant mass peak at 461.1099 Da in panel B (red) belongs to CPpY.

3.2 Cyclic voltammograms (CVs) of peptide-modified electrode in $[Ru(NH_3)_6]^{3+}$ solution

To demonstrate the feasibility of the method, we first investigated the voltammetric characteristics of phosphorylated and nonphosphorylated peptides in $[Ru(NH_3)_6]^{3+}$ solution. Fig. 3A shows the CVs of a gold electrode modified with nonphosphorylated peptide CPY and MCH in $[Ru(NH_3)_6]^{3+}$ solution at the scan rate ranging from 10 to 150 mV. A couple of redox peaks centred at –0.115 V was observed. The normalized reduction peak current plotted as a function of the square root of the normalized scan rate was obtained (cf. the inset in panel A), indicating the diffusion-controlled process. Fig. 3B shows the CVs acquired at CPpY/MCH-modified gold electrode. Two pairs of voltammetric peaks corresponding to the reduction and oxidation of $[Ru(NH_3)_6]^{3+}$ at the modified electrode are observed. The redox potential of the first peak (I) is close to that obtained at CPY/MCH-modified electrode, which is thus attributed to the diffusion-controlled wave. The second peak (II) with a post-current more negative than the diffusion-controlled current is the adsorption post-wave, since the normalized intensity of the post-peak shows a linear dependence with the normalized scan rate (cf. the inset in panel B). The reduction peak of the adsorption post-wave is higher that the oxidation peak, which is attributed to the stronger absorption capacity of negatively charged phosphate groups to $[Ru(NH_3)_6]^{3+}$ than to $[Ru(NH_3)_6]^{2+}$ [21,27]. For this, the signal characteristic of adsorbed $[Ru(NH_3)_6]^{3+}$ can be used to determine the ALP-induced change in the amount of phosphate groups.

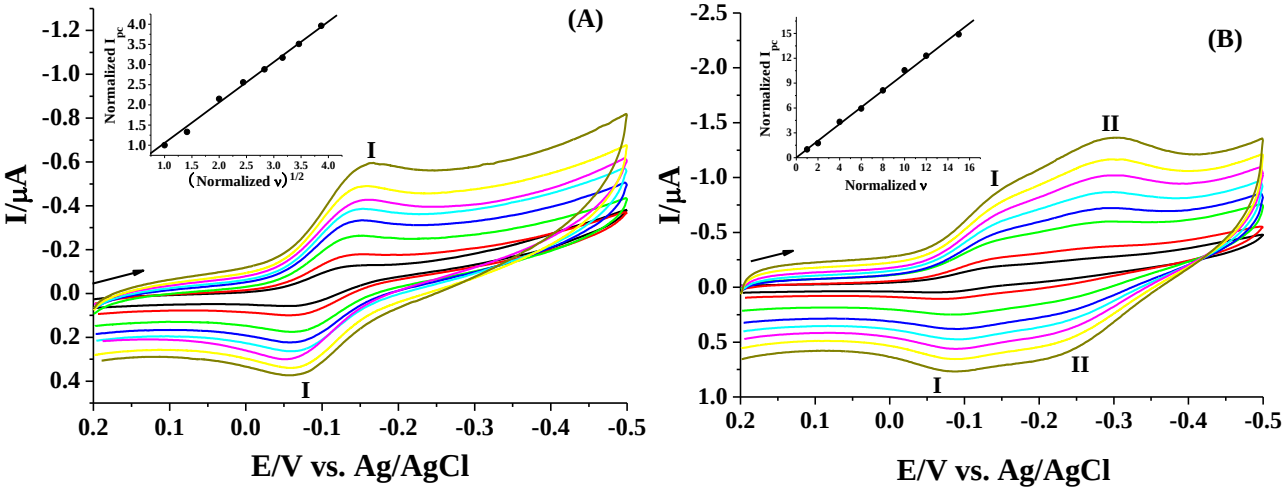

Figure 3. CVs acquired at CPY/MCH- (A) and CPpY/MCH- (B) covered electrodes in $[Ru(NH_3)_6]^{3+}$ solution at different scan rate (10, 20, 40, 60, 80, 100, 120 and 150 mV/s). The arrow indicates the scan direction.

3.3 Feasibility for ALP Detection

To explore the feasibility of the enzyme-assisted decrease in the current for ALP detection, we compared the CV responses of the gold electrode in $[Ru(NH_3)_6]^{3+}$ solution after incubation with the CPpY/ALP mixture. As shown in Fig. 4, no apparent adsorption post-wave was observed at the electrode after pretreatment with the CPpY/ALP mixture (red curve), implying that none or less amount of phosphate group-containing peptides were immobilized on the electrode. The control

experiment was conducted with thrombin instead of ALP in the same experimental condition. As a result, two pairs of voltammetric peaks were observed (blue curve), which is similar to that in the case of CPpY alone (black curve). The results indicated that the removing of phosphate groups is dependent upon ALP.

It is well known that the activity of enzyme is pH-dependent. The effect of pH on the enzymatic activity of the biosensors was therefore carried out. ΔIpc (the change of reduction current at –0.305 V (Ipc^0-Ipc'), where Ipc^0 and Ipc' represent the current in the absence and presence of ALP, respectively) was used here to evaluate the performance of the sensor. As shown in Fig. 5, ΔIpc reaches the maximum at pH 8.0, demonstrating that the optimal pH for the activity of the enzyme is 8.0. This value is also in agreement with the previous report [29]. Note that pH varying from 5.5 to 8.5 has no influence on the assembly of CPpY through Au-S bonds since no apparent difference in the post-current was observed in these pH regions (data not shown). Therefore, PBS buffer at pH 8.0 was chosen as the reaction media.

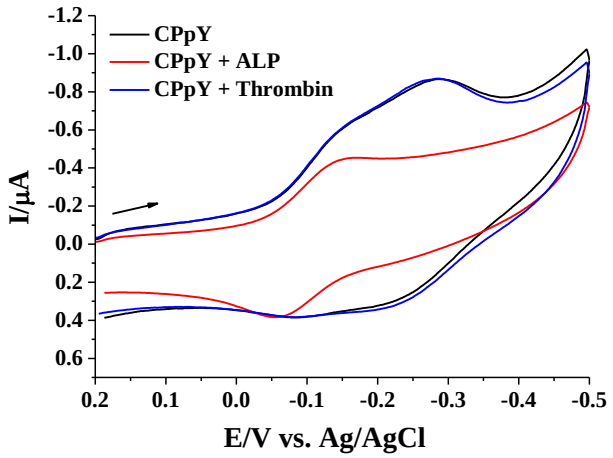

Figure 4. CVs acquired at the electrode pretreated with CPpY alone, CPpY/ALP and CPpY/thrombin before the modification of MCH. The scan rate was 100 mV/s. The concentration of both ALP and thrombin was 200 ng/mL.

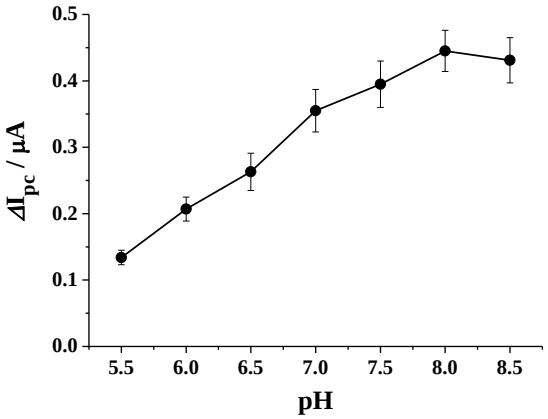

Figure 5. Effect of pH on ALP activity. The relative standard deviations (RSDs) from at least three replicates were shown as the error bars. The other experimental conditions were the same as those in Fig. 4.

3.4 Sensitivity to ALP

Another electrochemical technique, differential pulse voltammetry (DPV), can decrease the back ground charging currents and in turn increase the detection sensitivity. We evaluated the sensitivity of the proposed method using DPV by measuring ALP in a series of concentrations. Fig. 6 shows typical DPV responses of the electrode after incubation with CPpY and different concentrations of ALP. It is clearly seen that the post-current decreases accordingly with increased concentration of ALP, demonstrating that the addition of ALP at different concentrations to the CPpY solution results in the removing of different amounts of phosphate groups. The detection limit of this method was estimated to be 1 ng/mL, which is comparable to that achievable with other electrochemical strategies [11, 16]. However, our method obviates the use of expensive bioreagents, labeled target or nanoparticles for signal amplification, reducing the operation complexity.

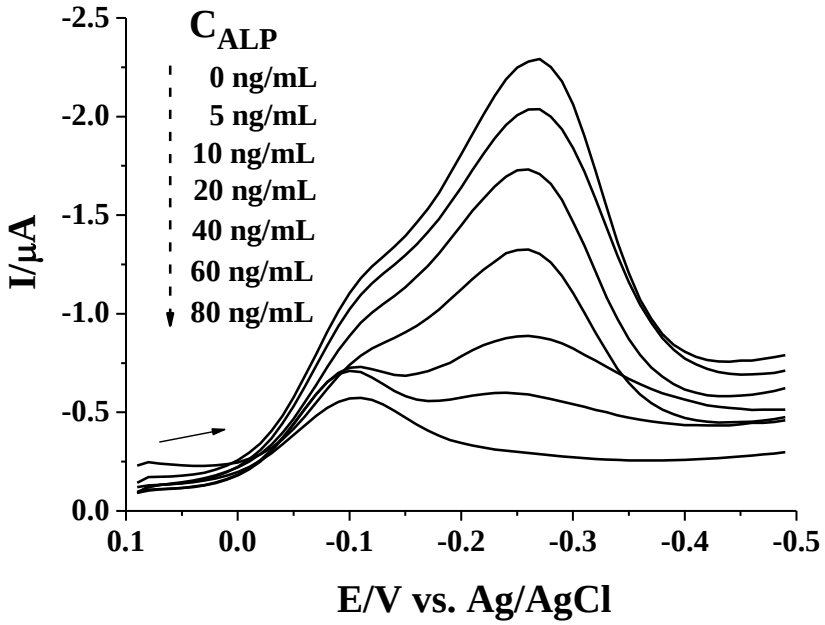

Figure 6. DPVs acquired at the electrode pretreated with CPpY and different concentrations of ALP before the modification of MCH. The other experimental conditions were the same as those in Fig. 4.

3.5 Assay of ALP activity

Levamisole is an ALP inhibitor that has been used in humans to treat parasitic worm infections, and has been studied in combination with other forms of chemotherapy for colon cancer, melanoma, and head and neck cancer [4, 30]. Unfortunately, the risk of serious side effects and the availability of more effective replacement medications have limited its therapeutical use [31]. Therefore, numerous levamisole analogs have been synthesized as anthelmintics and tested as inhibitors of ALP [4]. To demonstrate the feasibility of our method for screening the potential ALP inhibitors, levamisole was tested. As shown in Fig. 7, the addition of levamisole to the ALP solution prevented the ALP-induced

decrease in the current, indicating that the activity of ALP was inhibited by levamisole. The current increased significantly with increased inhibitor concentrations, indicating that the overall inhibition was more effective at higher inhibitor concentrations. Thus, the presented electrochemical method would be applicable to not only probing of ALP activity, but also screening of potential ALP inhibitors.

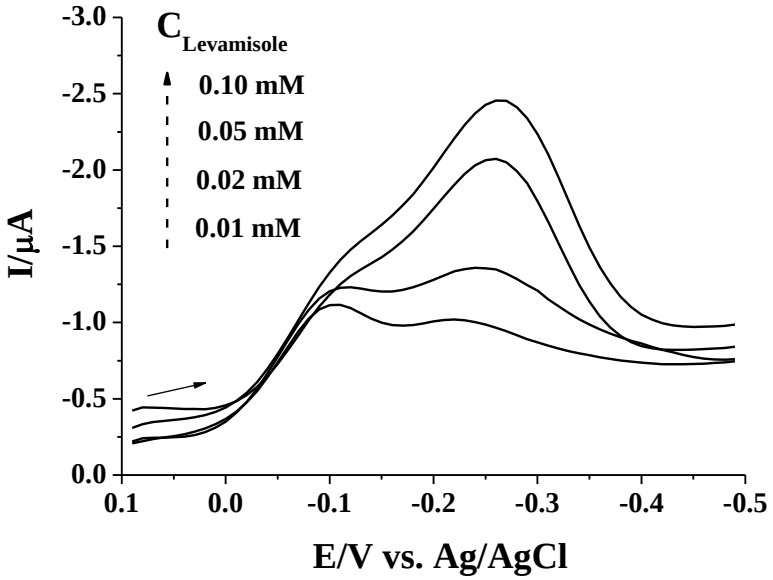

Figure 7. DPVs acquired at the electrode pretreated with CPpY/ALP in the presence of different concentrations of levamisole. The concentration of ALP was 80 ng/mL. The other experimental conditions were the same as those in Fig. 4.

4. CONCLUSION

In conclusion, we have demonstrated a simple, sensitive and label-free electrochemical sensor for probing of ALP activity and screening of ALP inhibitors. Compared with other electrochemical sensors, our method has following advantages: 1) the redox mediator, $[Ru(NH_3)_6]^{3+}$, is selected as the electrical signal readout, which is easy to prepare, convenient to operate with a good stability, and 2) the method obviates the use of expensive bioreagents, labeled target or nanoparticles for signal amplification, reducing the operation complexity. Moreover, we believe that the results will be valuable for the design of new types of biosensor for the label-free and sensitive detection of other enzymes (e.g. proteases and kinases).

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