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Investigation of the Interaction between Ferroceneboronic Acid and Sugars and Its Application in Probing of Enzyme Activity

Xiaojin Wang[*], *Lijiao Zhang*, *Fengyun Gu*, *Xiaojie Zhang*, *Ning Xia*[*]

College of Chemistry and Chemical Engineering, Anyang Normal University, Anyang, Henan 455000, People's Republic of China
[*]E-mails: xjwanghxx@sohu.com(X. W.); xianing82414@163.com(N. X.)

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Sugars play important roles in many biological processes, such as nutrition, metabolism, cell structure formation, and immunological protection. Phenylboronic acid (PBA) derivatives have been widely used as recognition molecules for sugars based on the formation of boronate ester bonds between boronic acid and 1,2-diol or 1,3-diol of sugars. In this work, ferroceneboronic acid (FBA, an electrochemically active ferrocene derivative) was used to investigate the interaction between boronic acid and phosphated as well as non-phosphated sugars. Based on the difference in the voltammetric responses of the complexes between FBA and examined sugars, FBA probe was used to monitor the activity of enzymes, such as phosphoglucose isomerase (PGI) and alkaline phosphatase (ALP). This work would be valuable for understanding of the diol-boronate interaction and screening of new enzyme inhibitors.

Keywords: Ferroceneboronic acid; sugars; phosphoglucose isomerase; alkaline phosphatase

1. INTRODUCTION

Sugars are important biological molecules because they are essential in many biological processes, such as nutrition, metabolism, cell structure formation, and immunological protection [1]. For example, glycolysis, the metabolic pathway that converts glucose into pyruvate, releases the free energy released for the formation of the high-energy compound adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH) [2]. The first step in this process is phosphorylation of glucose by a family of enzymes called hexokinases to form glucose 6-phosphate (G6P). Then, G6P is re-arranged into fructose 6-phosphate (F6P) by phosphoglucose isomerase (PGI, EC 5.3.1.9) (Fig. 1A) [3]. Conceivably, the conversion of G6P into F6P by PGI plays an important role in the regulation of carbohydrate metabolism [4]. It was reported that PGI activity is responsible for

many diseases. For example, deficiency in PGI activity in human leads to non-spherocytic hemolytic anemia, and high levels of PGI activity are measured in the sera of patients with certain cancers [5-7]. PGI has also been studied as a potential target for the design of novel inhibitors against parasites because of the differences between human and parasitic glycolytic pathways and the dependence of the parasite on glycolysis [8,9]. Furthermore, phosphorylation and dephosphorylation play pivotal roles in cellular regulation and signaling processes. Alkaline phosphatase (ALP, EC 3.1.3.1) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins and phosphated sugars (Fig. 1A) [10-12]. In humans, ALP is present in all tissues throughout the entire body. This enzyme is also commonly used in clinical assays and acts as biomarker in the diagnosis of many diseases, including bone diseases, liver dysfunction, prostatic cancer and bile duct blockage [13-15]. The normal levels of ALP may differ by gender and age. Currently used methods for probing of the activity of PGI and ALP include tandem mass spectrometry and spectrophotometric detection [10,16,17]. However, these methods are time-consuming and need complicated instruments. Therefore, simple procedure for probing of the activity of these two enzymes and screening of potential enzyme inhibitors has important clinical implications.

Boronic acids are well known to bind the 1,2-diol and 1,3-diol of sugars under aqueous conditions. Phenylboronic acid (PBA) derivatives that have a boronic acid moiety can be thus used as recognition molecules for sugars. Recently, much attention has been devoted to the construction of electrochemical and optical sugars sensors based on the interaction of sugar and boronic acid [18,19]. The sugar-induced changes in the UV–visible or fluorescence spectra and the current or potential of PBA derivatives are caused by the formation of cyclic bononate ester covalent bond. Among kinds of boronic acid-based sensors, electrochemical technique is simple, sensitive, and inexpensive to implement. Ferroceneboronic acid (FBA), an electrochemically active ferrocene derivative, can bind to sugars. Many reports have also suggested the interaction of FBA and diol-containing species [20-24]. For example, Chien et al. reported an amperometric biosensor for the determination of fructosyl valine (a diagnostic target for type 2 diabetes) using FBA [20]. Moore and Wayner studied the effect of pH on the redox properties of the FBA-sugar complex in aqueous buffers [21]. Lacina and Skládal investigated the complexation interaction of FBA and a set of saccharide molecules and determined the corresponding affinity equilibrium constants [22]. However, to the best of our knowledge, there is no report on the interaction of FBA and phosphated sugars, such as G6P and F6P. In the present work, we investigated the interaction between FBA and G6P, F6P, glucose, fructose as well as fructose-1,6-diphosphate (FDP), and reported a simple and rapid electrochemical method for probing of the activity of enzymes, such as PGI and ALP.

2. EXPERIMENTAL

2.1 Chemicals and reagents

Ferroceneboronic acid (FBA) and glucose phosphate isomerase (PGI, EC 5.3.1.9) were obtained from Sigma–Aldrich. Glucose, fructose, levamisole, alkaline phosphatase (ALP, EC 3.1.3.1)

and fructose-1,6-diphosphate (FDP) were purchased from Sangon Biotech. Co., Ltd. (Shanghai, China). Glucose-6-phosphate (G6P) and fructose-6-phosphate (F6P) were provided by Ebioeasy Company (Shanghai, China). All other reagents are of the AR grade. FBA stock solution was prepared with phosphate-buffered saline solution (PBS buffer, 200 mM, pH 7.0) containing 1% DMSO. The ALP and sugars stock solutions were prepared with water. All aqueous solutions were prepared with a Millipore system (Simplicity Plus, Millipore Corp.).

2.2 Electrochemical measurements

The glass carbon (GC) electrode with a diameter of 3 mm was polished with alumina power, and then sonicated in ethanol and water. Voltammetric determination in phosphate-buffered saline solution (PBS buffer, 200 mM) was performed on an uECS-PRO electrochemical workstation (Changchun Institute of Applied Chemistry Chinese Academy of Science, China) using a homemade plastic electrochemical cell room temperature. A platinum wire and a Ag/AgCl electrode were used as the auxiliary and the reference electrodes, respectively. For the conversion of G6P to F6P, PGI (10 mg mL^{-1}) was incubated with 50 mM G6P solution (pH 7.0) for 30 min at 25 °C. Then, 20 μL of the PGI/G6P solution was mixed with 80 μL FBA solution for 30 s before the electrochemical measurement. For the assay of ALP activity, ALP was incubated with 1 M F6P solution at 25 °C. The pH of F6P solutions was adjusted to the desired value with NaOH/HCl before the addition of ALP. For the electrochemical measurement, 20 μL of the ALP/F6P mixture was incubated with 1.98 mL FBA solution for 30 s.

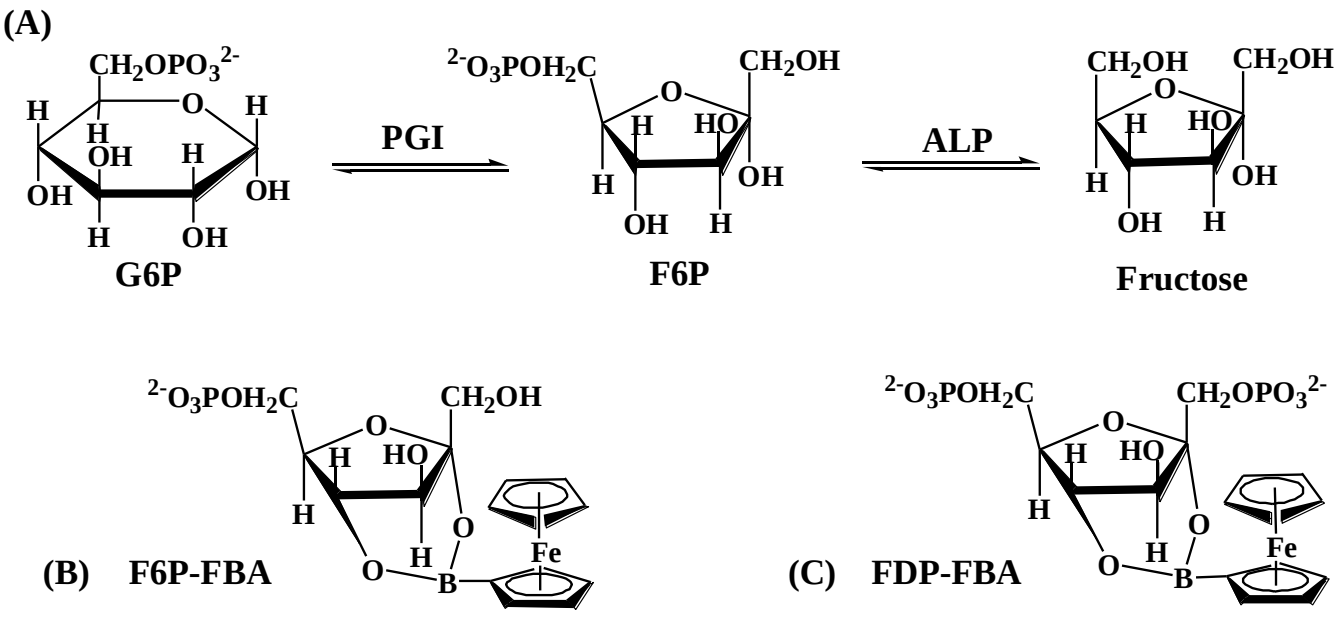

Figure 1. Isomerization of G6P to F6P by PGI and dephosphorylation of F6P by ALP (A) and speculative structure of the complexes between FBA and F6P (B) as well as FDP (C).

3. RESULTS AND DISCUSSION

3.1 Interaction of FBA with phosphated and non-phosphated sugars

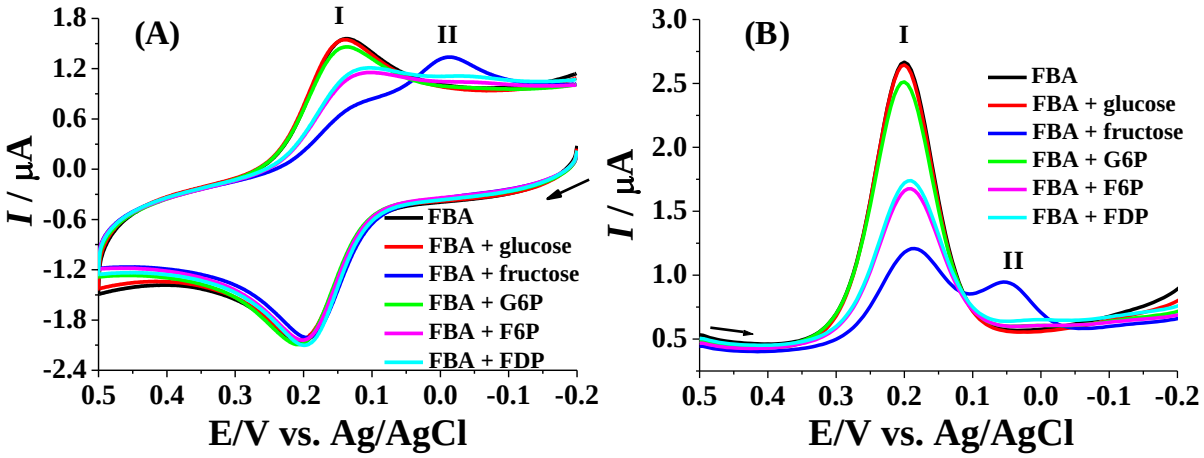

Figure 2. Cyclic voltammograms (CVs) (A) and differential pulse voltammograms (DPVs) (B) of FBA in the absence and presence of diverse sugars. The final concentrations of FBA and sugars were 100 μM and 10 mM, respectively. The scan rate in panel A was 100 mV/s and the arrow indicates the scan direction. Pulse height and pulse width in panel B were 50 mV and 50 ms, respectively.

The electrochemical behavior of the FBA-sugar was probed by cyclic and differential pulse voltammetry. As shown in Fig. 2A, the CV of FBA shows a couple of redox peaks with Epa = 0.21 V and Epc = 0.18 V (peak I). Interestingly, the addition of fructose to the FBA solution induced the appearance of a new reduction peak at −0.02 V (peak II), which is attributed to the reduction of the FBA-fructose complex. No obvious change was observed after the addition of glucose or G6P at the concentration below 10 mM, which is due to the poor binding affinity of boronic acid to glucose than to fructose [22,25]. The reduction peak of FBA decreased markedly after the addition of F6P or FDP. Note that no obvious change in the oxidation peak was observed after addition of these sugars. This is attributed to the higher affinity of the oxidized form of FBA to fructose than the reduced one [21], which can generally be explained as follows: positive charge of the ferrocenium moiety favors the interaction of boron with additional hydroxy group to form a tetrahedral configuration. However, differing from the FBA-fructose complex, no shift in the reduction peak of F6P-FBA was observed. This is probably because the negatively charged phosphate group in F6P inhibits the formation of tetrahedral boronate etster. Moreover, the voltammetric response of FDP-FBA was same as that of F6P-FBA, indicating that the phosphorylation at 1’ position did not prevent the binding of boroinc acid to diol group, that is, FBA interacts with F6P or FDP through the binding between boron and cis-diol at 2’ and 4’ hydroxyl groups (Fig. 1B and 1C). Differential pulse voltammetry can decrease the background charging currents and in turn increase the detection sensitivity. We also investigated the potential/current change of FBA induced by these examined sugars. The results, shown in Fig. 2B, are consistent with those achievable by CV.

3.2 Feasibility for probing of PGI activity

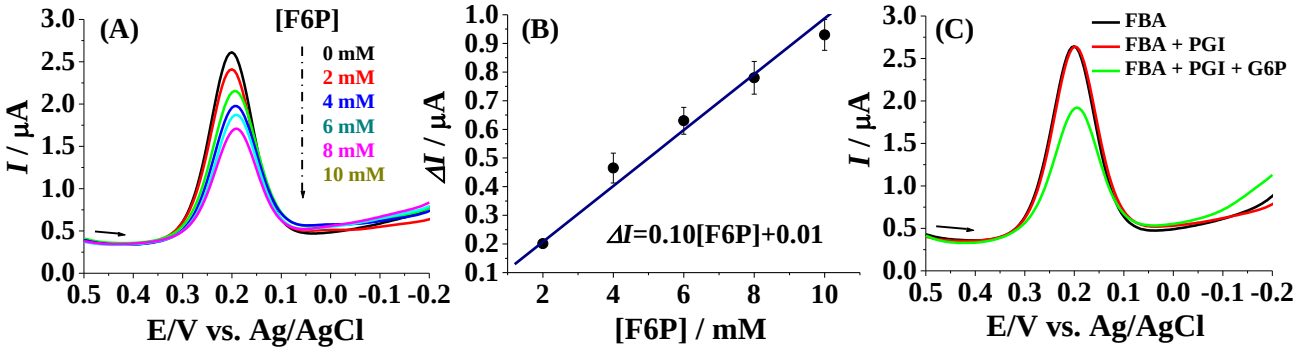

Figure 3. (A) DPVs of FBA in the presence of different concentrations of F6P. (B) Plots of ΔI against the F6P concentration. Each point was averaged from three replicates, and the absolute error deviations are shown as the error bars. (C) DPVs of FBA in different systems. The other experimental conditions are the same as those in Fig. 2.

Because the potential or current of FBA was different after binding to the individual examined sugars, we assumed that these diverse sugars would be determined on this basis and the results could found many applications in probing the activity of relevant enzymes. For example, as mentioned in Introduction, G6P is re-arranged into F6P by phosphoglucose isomerase (PGI) (Fig. 1A). In view of the difference of voltammetric response between G6P-FBA and F6P-FBA, we suggested that this method could be used for probing PGI activity. We first evaluated the dependence of the current change upon F6P concentration. Fig. 3A depicts the voltammetric responses in FBA solutions containing different concentrations of F6P. The total concentration of G6P and F6P in the assay kept at 10 mM. The current decreased with the increase of F6P concentration. ΔI (ΔI = I_0 − I, where I_0 and I represent the current value in the absence and presence of F6P, respectively) was used here to evaluate the performances of the sensor. As shown in Fig. 3B, ΔI increases linearly with the increase of F6P concentration, which can be expressed using ΔI = 0.10 [F6P] + 0.01 (R^2 = 0.99). As shown in Fig. 3C, the addition of the G6P/PGI mixture induced the significant decrease in the current of FBA (green curve), which is attributed to the formation of the complex between FBA and F6P resulting from the PGI-mediated isomerization of G6P since PGI alone did not cause apparent change in the voltammetric response of FBA (red curve). The results indicated that probing of the PGI activity with FBA as the redox probe is possible. The equilibrium conversion of G6P to F6P was calculated to be 53 % by the calibration curves.

3.3 Probing of ALP activity

As shown in Fig. 2, different voltammetric responses were observed after addition of fructose and F6P to the FBA solutions. ALP can remove the phosphate group from many types of molecules, including phosphated sugars (Fig. 1A) [10-12]. We therefore assumed that the activity of ALP could be monitored with FBA as a redox probe. The voltammetric responses of FBA in the presence of

different concentrations of fructose and F6P were shown in Fig. 4A. The total concentration of fructose and F6P was 10 mM. I_{II}/I_I, the ratio of the reduction current of peak II (I_{II}) to that of peak I (I_I), was used to evaluate the performance of the sensor. As show in Fig. 4B, the I_{II}/I_I ratio increases linearly with the increase of fructose concentration, which can be expressed using $I_{II}/I_I = 0.07$ [Fructose] − 0.01 ($R^2 = 0.99$). Expectedly, after addition of the mixture of ALP and F6P, the electrochemical response reveals a reduction peak (peak II), which is attributed to the complex between FBA and fructose resulting from the dephosphorylation of F6P (cf. red and blue curves in Fig. 4C). Therefore, probing of the ALP activity with FBA as the redox probe is also possible since ALP did not cause apparent change in the voltammetric response of FBA (cf. black curves in Fig. 2B and Fig. 4C).

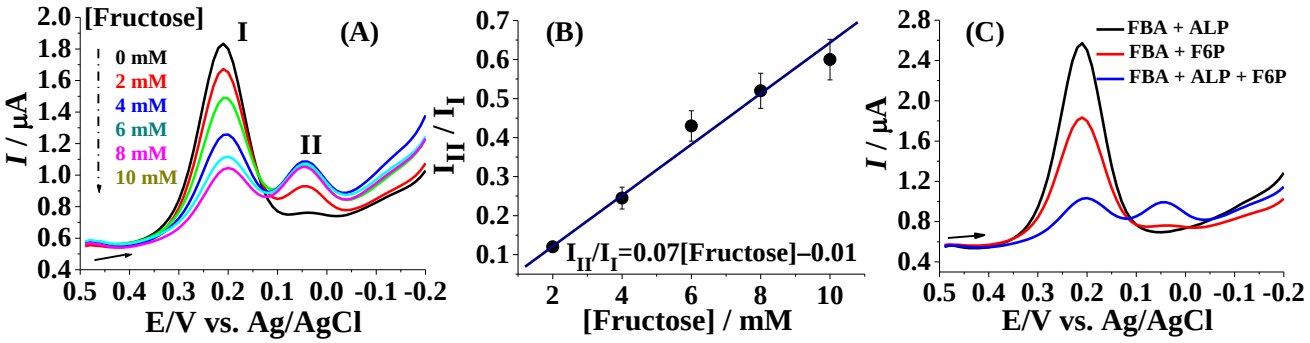

Figure 4. (A) DPVs of FBA in the presence of different concentrations of fructose. (B) Plots of the I_{II}/I_I ratio against the fructose concentration. (C) DPVs of FBA in different systems. ALP (50 μg mL^{-1}) was pre-incubated with F6P in pH 8.0 PBS solution for 30 min. The other experimental conditions are the same as those in Fig. 2.

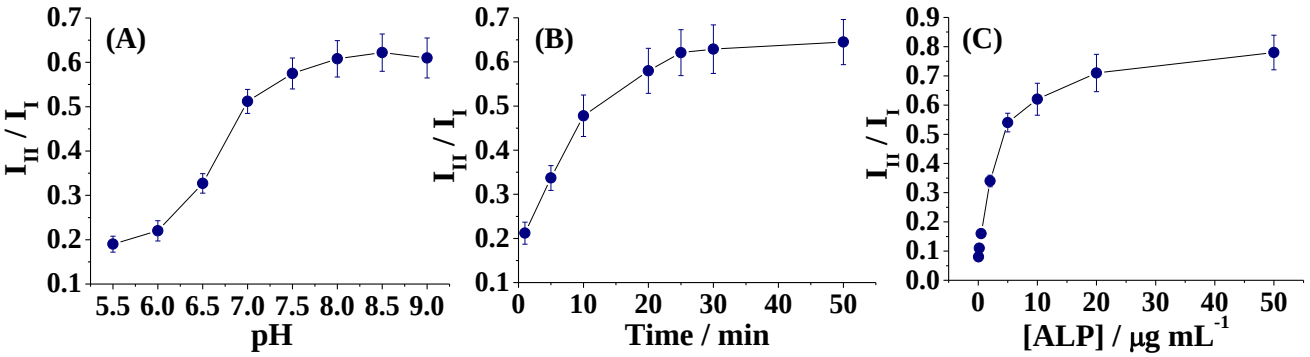

Figure 5. Effect of pH (A), incubation time (B) and final ALP concentration (C) on the III/II ratio. The concentrations of ALP were 10 μg mL^{-1} in panels A and B. The incubation time was 30 min in panels A and C. The pH value was 8.5 in panels B and C.

It is well known that the activity of enzymes is pH-dependent. We also evaluated the effect of pH on the activity of ALP by pre-incubating the F6P/ALP mixed solution at pH varying from 5.5 to 9.0. As shown in Fig. 5A, the I_{II}/I_I ratio reaches the minimum at pH 8.5, indicating that the optimal pH

for the activity of ALP is around 8.5. Therefore, PBS buffer at pH 8.5 was chosen as the reaction media for the detection of ALP concentration and activity. The relationship of I_{II}/I_I and incubation time was also investigated. As shown in Fig. 5B, I_{II}/I_I increases with the increase of the incubation time, and begins to level off beyond 25 min, indicating that ALP-catalyzed dephosphorylation reaction is completed within 25 min. Moreover, we investigated the dependence of I_{II}/I_I upon ALP concentration under the optimal conditions. As shown in Fig. 5C, I_{II}/I_I increases with the increase of ALP concentration. The detection limit of this method was estimated to be 8 ng mL^{-1}.

3.4 Feasibility for screening of ALP inhibitor

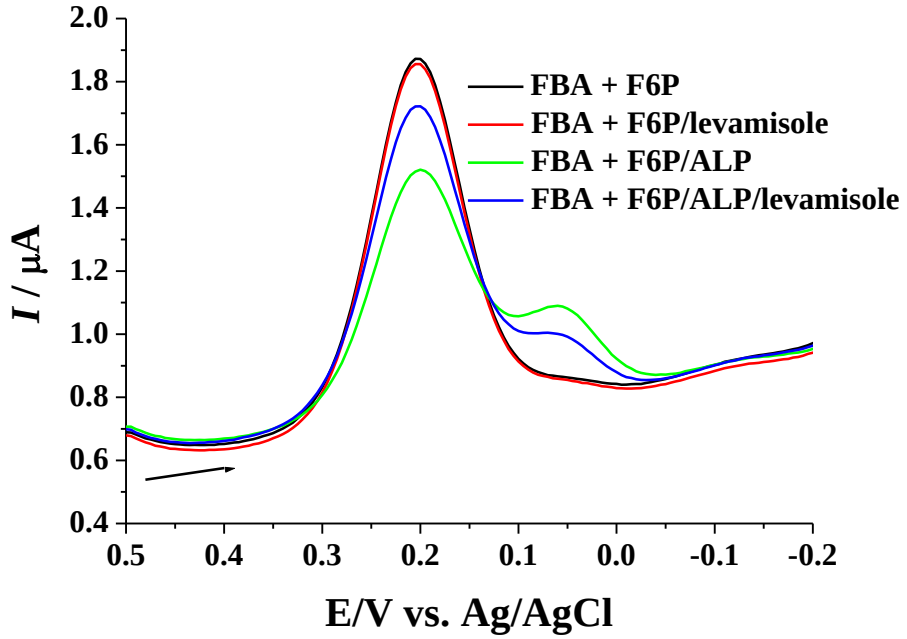

Figure 6. DPVs acquired at FBA solutions with the addition of F6P, F6P/levamisole, F6P/ALP and F6P/ALP/levamisole. The final concentrations of ALP and levamisole were 1 μg mL^{-1} and 0.1 mM, respectively. The other experimental conditions are the same as those in Fig. 4.

ALP inhibitors have also been shown to be promising drugs for curing the diseases caused by ALP-overexpression. Moreover, ALP has become a useful tool in molecular biology laboratories as an enzymatic label [26]. Therefore, simple and sensitive method for probing of ALP activity and screening of potential ALP inhibitors has important clinical implications. For example, levamisole, an ALP inhibitor, 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. Unfortunately, the risk of serious side effects and the availability of more effective replacement medications have limited its therapeutical use [27,28]. Therefore, numerous levamisole analogs have been synthesized as anthelmintics and tested as inhibitors of ALP [28]. To demonstrate the feasibility of our method for screening the potential ALP inhibitors, levamisole was tested. As shown in Fig. 6, the addition of levamisole to the ALP/F6P mixed solution prevented the appearance of the new peak (peak II), indicating that the ALP-catalyzed dephosphorylation of F6P was inhibited by levamisole.

Thus, the presented electrochemical method could not only be applied in probing of the ALP activity and but also in screening of the potential ALP inhibitors.

4. CONCLUSION

In summary, we investigated the interaction between FBA and phosphated and non-phosphated sugars. After binding to the diverse sugars, FBA showed different voltammetric responses. Based on the change in the potential/current of FBA induced by these examined sugars, we suggested that FBA can be used as a redox-active probe for monitoring the activity of enzymes, such as PGI and ALP. To demonstrate the application of this method in screening of enzyme inhibitors, the effect of levamisole (a known inhibitor of ALP) on the activity of ALP was investigated. We believe that the method would find many applications in laboratory research because it requires very simple and security sample-handling-procedure and minimum instrumental investment.

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