

## Development of a Novel Electrochemical Monitoring Method of Enzymic Hydrolysis

Xingyue Wei\*, Lin Zhuo

College of Environment and Resources, Chongqing Technology and Business University, Chongqing 400067, P.R. China

\*E-mail: [xingyuewei\\_cq@yeah.net](mailto:xingyuewei_cq@yeah.net)

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In this work, we assessed the electrochemical behaviour of the products of substrates of the enzyme label, alkaline phosphate, ordinarily adopted into the field of electrochemical immunosensors. Cyclic voltammetry (CV) as well as amperometry of such resultants were respectively conducted at glassy carbon (GC) and gold (Au) electrodes. With mouse IgG to be a model, an ALP enzyme-magnified amperometric immunosensor with a sandwich shape came into being. Such immunosensor worked through the electropolymerization of *o*-aminobenzoic acid (*o*-ABA) polymer with conductivity on Au as well as GC electrodes' appearance. Then the anti-mouse IgG adhered to the electrode appearance by covalent bonding between IgG antibody and the carboxyl species from poly(*o*-ABA). When 2-phospho-l-ascorbic acid was adopted to be a substrate, the most optimized signal could be generated through the poly(*o*-ABA)/Au immunosensor, indicating amperometric immunosensors with the basis of a conductive polymer electrode system were of great sensitivity to concentrations of the mouse IgG down to 1 ng/mL.

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**Keywords:** Electrochemistry; Enzymic hydrolysis; Immunosensor; Electrode; Mouse IgG

### 1. INTRODUCTION

Over the past years, immunoassay has become a significant method for the purpose of analysis. To be detailed, enzyme immunoassay is such a method working through the quantitative determination of existing antigen or antibody in the to-be-analysed material, the determination is based on a certain immuno-interaction, which can be measured by monitoring the performance of enzymes labels attached to the target antigen or antibody. These labels have a major benefit in that the magnification of signal can be achieved because the resultant molecules labelled by every enzyme are of great production. In this field, alkaline phosphatase is one of the typical labels [1]. Being conveniently

attached to proteins such as antibody and hapten, ALP witnessed no less than seventy years of investigation. In addition, it has a great yield and can be applied to a great number of substrates.

Detecting phosphate in water source's appearance is significant in that it can assess how nutrient transforms in the aspects of ecology and biogeochemistry. Moreover, it is applicable to the issues concerning regulation and legislature, which indicates inland channel's eutrophication resulting from human activities like agriculturally exploring has been focused [2-7]. Recently, one of the British administrations, the Environment Farming and Rural Affairs, issued relative statistics, indicating that the phosphate concentrations are over 0.1 ppm for more than sixty percent the British waters measured in length. Besides, basically reasons concerning agriculture account for forty percent of phosphate's increase domestically. Clearly, the above data is diverse according to different regions and seasons [8, 9]. However, it is of vital importance to investigate elements like phosphate for balancing and sustaining the waters. It is pretty difficult to detect the wide spread materials in aspect of agriculture that contain superlative phosphate. Arguably, measurements with the basis on field could function as a precious and elastic choice for screening.

The application of such measurements is appealing in that they can offer suggestive measurements for larger areas, but regardless of derivation factors such as delays, costs, and sample integrity related to conventional analysing ways on the basis of laboratory. Being able to supply fast detection, such approaches are beneficial as for on-site assessment, while real situations must be taken into account. Although various sets of colorimetric spot tests that are pretty portable could be purchased, such methods are easy to be intervened, just producing nature determination as the best outcome. Detecting with electrochemical approaches could cope with the fast as well as quantitative field assessment which could be handled by researchers except for experts. Diverse detecting devices adopted in the field of decentralised sensing could be miniaturized, portable, and simple for operation [10]. It would be practical to infer that the above mentioned techniques could bring about technological future for phosphate's site testings, while selective as well as sensitive matters have to be made clear [10-12].

As for immunosensors adopted in the field of electrochemistry, an ALP enzyme has been applied for producing organic resultants with electro-activity. Through detection, the majority of such products could be quantitatively assessed. On an ALP substrate, the redox reaction of the resultants generated through the enzyme hydrolysis could be normally detected rapidly with such detector. With the engagement of ALP substrates, in order to achieve electroanalysis in immunoassays, there are other substrates like catechol monophosphate [13], 3-indoyle phosphate (IP) [14], hydroquinone diphosphate (HQDP) [15], 4-nitrophenol phosphate (NPP) [16], *p*-aminophenyl phosphate (APP) [17], 1-naphthyl phosphate (NTP), phenyl phosphate (PheP) as well as 2-phospho-l-ascorbic acid (AAP). Through enzyme reaction, such substrates are respectively transformed to the electroactive groups catechol, indigo carmine (IC), hydroquinone (HQ), 4-nitrophenol (NP), 4-aminophenol (AP), 1-naphthol (NT), phenol (Phe), and l-ascorbic acid (AA) [18-25].

This work makes a comparison between diverse substrates as well as the resultants generated through ALP enzymatic reaction, in order to develop an electrochemical immunosensor with high sensitivity. Under circumstances with no differences, amperometry as well as CV with triple electrode samples like Au and GC were adopted to research 7 products of substrates during ALP reaction.

## 2. EXPERIMENTS

HQ, NP, acetic acid, 11-mercaptopundecanoic acid, as well as sodium acetate were commercially available in Aldrich, while AP, AAP, as well as 6-mercapto-1-hexanol could be purchased from Fluka. Sigma was the place where Phe, IC, NT, AA, ethanolamine-HCl, *o*-ABA, sulfuric acid, Tris-hydrochloride (Tris), sodium chloride (NaCl), Tween 20, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC), *N*-hydroxysulfosuccinimide (NHS), bovine serum albumin fraction V (BSA), mouse IgG (serum as the source), anti-mouse IgG antibody (produced in goat), as well as ALP anti-mouse IgG antibody processed through conjugation (goat as the source) could be commercially obtained. The autoclaved water processed through double-deionization (18.2 MΩ cm) was applied for preparing the stock and buffer solutions needed in the experiment.

Located in the United States, a CHI 660 electrochemical workstation was founded in CH Instruments. Functioning as a laboratory, it provided a 1.5 mL of traditional triple-electrode cell in the field of electrochemistry for conducting the whole set of electrochemical immunoassay at ambient temperature. Measurements concerning amperometric as well as CV adopted such a set, together with the counter electrode with platinum wire, as well as CH Instruments' Ag/AgCl reference electrode. As for the functioning electrode, a Glassy Carbon electrode, CH Instruments' 3-mm-diameter GC disk electrode as well as its 2-mm-diameter Au disk electrode have been applied, not the same time. The amperometric experiment had the approach of magnetic mixture conducted.

The measurements concerning electrochemistry were conducted under ambient temperature. In terms of AA, AP, HQ, IC, NT and Phe, the experiment adopted the 0.5 M Tris buffer solution whose pH was 8.5. Meanwhile, as to NP, the experiment used a 0.1 M acetate buffer solution with a pH of 4.6. CV detected diverse resultants' reactions on various electrode samples. Those voltammograms that correspond with this CV have been also filed. Besides, this CV was adjusted with a scanning rate of 100 mV s<sup>-1</sup>. The electrolyte of 1.0 mL adopted in CV measuring method has been applied to hydrodynamic amperometric measurements in mixture. The background current having gradually become stable, every ALP substrate's equal share was put in, with the current responses accordingly filed to be a function of time.

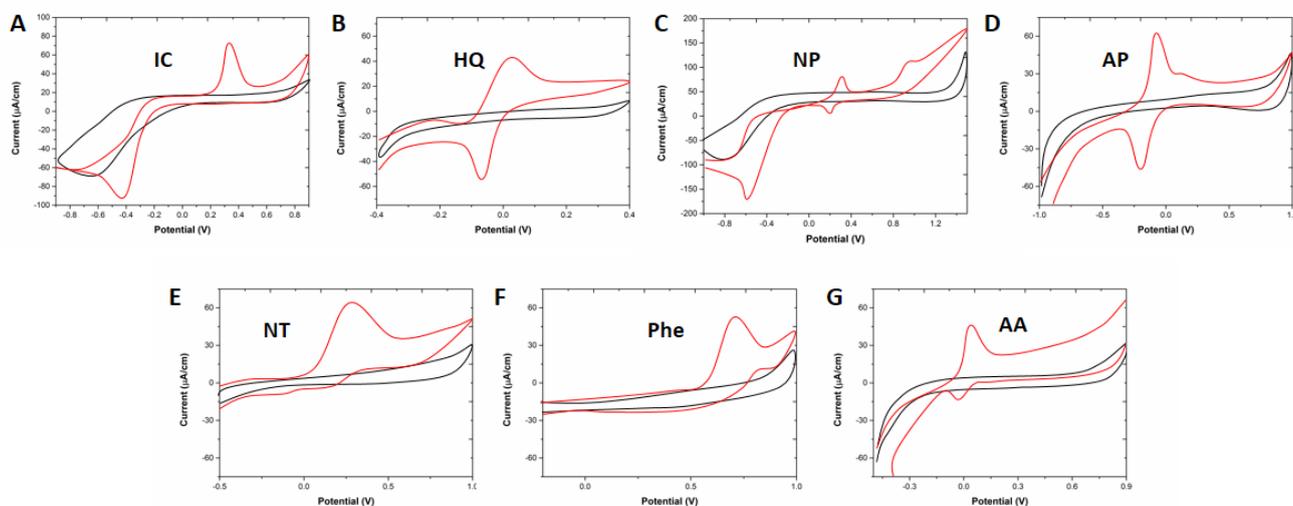
## 3. RESULTS AND DISCUSSION

Electrochemical adaptations depend on the situation where the hydrolysis resultants are oxidized selectively as well as sensitively. The nitrophenolate systems have been mainly adopted in the field of spectroscopic detection while being available in an electrochemical way. Nevertheless, it has disadvantages. The potentials have to be pretty powerful for the oxidization of phenol constituent. In addition, under peroxide measuring process, they could intervene the oxidization of the remaining constituents existing in the same sample. What is more, this method becomes more likely to be less sensitive and less likely to continue reproduction, since there are polymeric deposits brought about by the oxidization on the electrode to impede the corresponding electrode. To address this problem, the

needed potentials have to be lower, thus it is advisable to choose another label that needs relatively lower potentials to be oxidized.

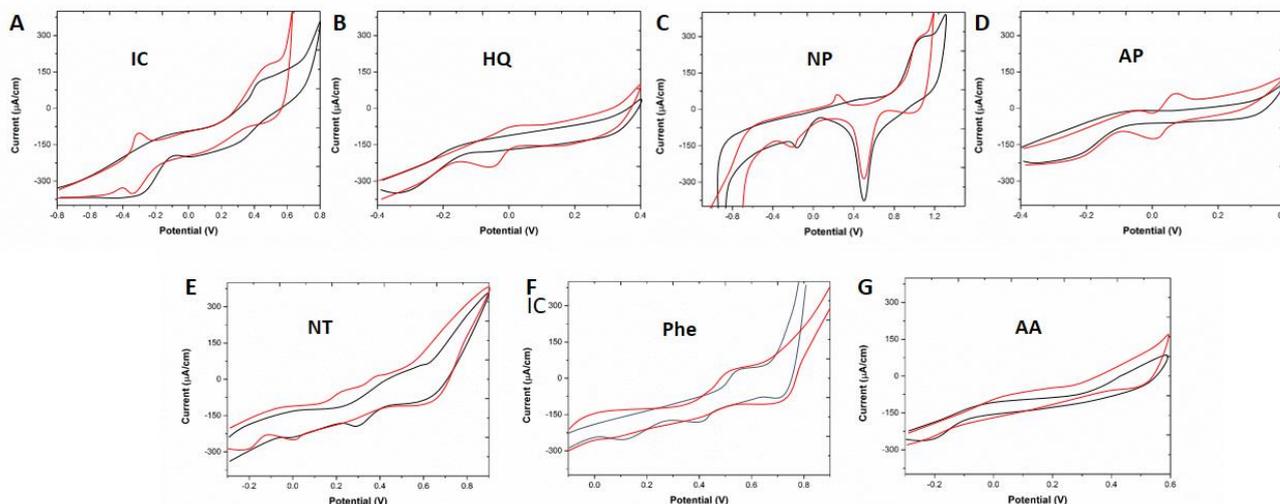
If the solution is alkaline, alcoholic (ROH) products (IC, HQ, NP, AP, NT, Phe, and AA) can be obtained from phosphate ester ( $\text{RHPO}_4^-$ ) functional group of its substrates (IP, HQDP, NPP, APP, NTP, PheP, and AAP) correspondingly through the hydrolysis of ALP. Such resultants could subsequently be detected in an electrochemical way.

ALP's substrate ought to generate no electrochemical signal if the potential is the same as the other form that has been processed through dephosphorylation. Having a broad substrate specificity and high turnover, this enzyme has been largely investigated as an enzyme label for electrochemical assays [26, 27]. The electrochemical behaviours of seven most commonly used products named Being usually adopted, the resultants of AA, AP, HQ, IC, NP, NT, and Phe have their electrochemical performances researched by means of CV and amperometry at GC and Au electrodes that have not been treated through modification. Fig. 1A to 1G demonstrate CV recorded using Au as well as GC electrodes correspondingly. To be specific, diverse resultants on one electrode sample perform in diverse ways in the aspect of electrochemistry. Meanwhile, on diverse electrode samples, one resultant performs in diverse ways. Compared with the electrode of GC, background CV signals on Au are much higher. This observation may be explained by the following. The applied current causes oxidation of NP, which can then react with the thiocholinc produced upon hydrolysis [28]. At low currents, little iodine is formed, so the change in potential with Such a phenomenon could be due to stronger conductivity of Au than that of GC. In this way, there would be more two layers on Au. Thus, Au electrode gained relatively big background current. The cyclic voltammogram in and Fig. 2, the provided the highest anodic current density of  $49.61 \mu\text{A}/\text{cm}^2$  at anodic potential peak at  $0.66 \text{ V}$  at Au electrodes, respectively.  $50.41 \mu\text{A}/\text{cm}^2$  IC's maximum anodic current density, has been achieved at  $0.30 \text{ V}$  with GC electrode. In terms of GC, AA's bottom anode potentials came into being at  $0.01 \text{ V}$ . Meanwhile, as to Au electrodes, AP's anode potentials reached the bottom at  $0.07 \text{ V}$ .



**Figure 1.** (A) Cyclic voltammograms and of diverse substances at GC. (A) IC; (B) HQ; (C) NP; (D) AP; (E) NT; (F) Phe and (G) AA.

In the process of ALP reaction, IB, namely indigo blue, could be produced by IP substrate through enzymatic hydrolysis. IB performs poor in solubility in water solution. Thus, the water was infused with fuming sulphuric acid to get IC with stronger solubility for compensation. However, in terms of substrate, IP cannot be considered good, since detecting IC through electrochemical way is pretty complex. On the contrary, because of the reaction in chemistry that is not reversible, AA, NT and Phe just show its oxidization peak. CVs of NP, NT and Phe indicate less reversible as for overall oxidization researches [29, 30].

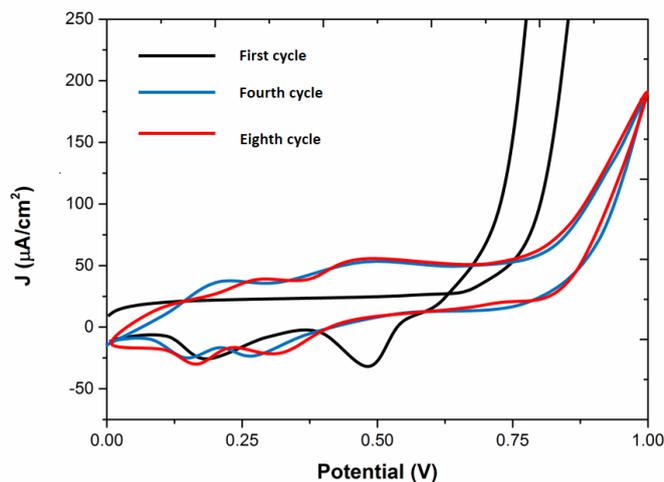


**Figure 2.** (A) Cyclic voltammograms of diverse substances at Au electrode. (A) IC; (B) HQ; (C) NP; (D) AP; (E) NT; (F) Phe and (G) AA.

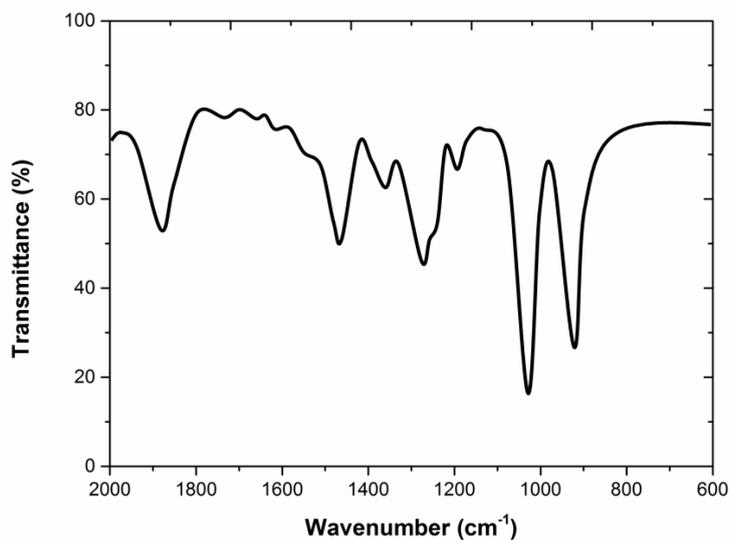
They shared some similarities as for electrochemical reactions. The solid line of them indicated the electrode fouling, which happened at the same time with the absence of amperometric reactions. On the appearance of electrode, the electroinactive products accumulate due to the electrical oxidation of NP, NT as well as Phe. Besides, the electrode became inert because the polymer was generated on it. Being inert made it less applicable to the electrochemical immunosensors. For AA, cyclic voltammograms showed was nearly constant indicating that less passivation occurring at all of electrodes.

Through CV, the solution of *o*-ABA within which there is  $\pi$  electron backbone went through electrical polymerization with potential ranging from 0–1 V to 40 mV/s. As indicated in Fig. 3, the first, the sixth, and the eighth cycle of *o*-ABA CV electrical polymerization demonstrating voltammogram became pretty rapid among the top of six cycle, preventing the electron transfer between the electrode and the electrolyte [31]. Therefore eight cycles were chosen for electrical polymerization of such sandwich immunoassay system. Fig. 4 shows the FTIR spectrum of the poly(*o*-ABA). It can be seen that the copolymer and poly(*o*-ABA) shows a new characteristic peak appeared at  $1689\text{ cm}^{-1}$ , which is ascribed to the strong stretching vibration of carboxyl groups. Several other important characteristic absorption bands at  $1593$ ,  $1517$ ,  $1309$ , and  $1242\text{ cm}^{-1}$  are consistent with those absorption bands which allow the identification of polyaniline: benzenic-quinonic nitrogen, C—C

aromatic, aromatic amine, and C—N—C. The assignments of other characteristic peaks have been reported in the previous studies [32, 33].



**Figure 3.** Cyclic voltammogram of *o*-ABA electropolymerization at Au electrode: 1st cycle; 6th cycle and 8th cycle with the scanning rate of 40 mV/s.

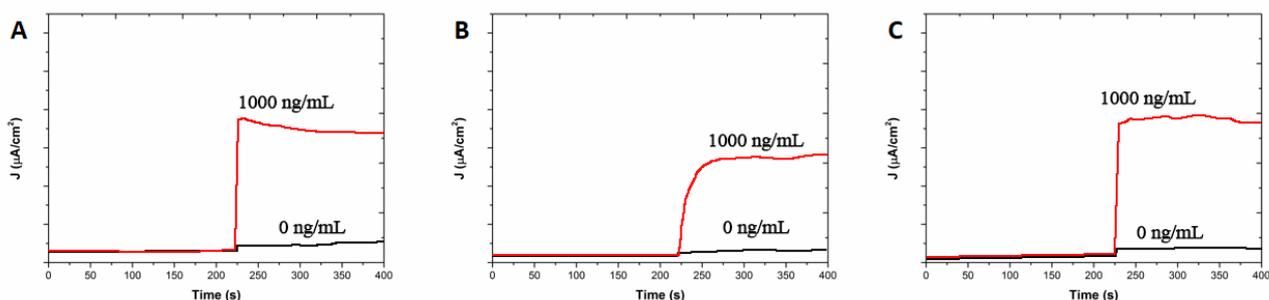


**Figure 4.** Total internal reflection FTIR spectrum of poly(*o*-ABA).

As for preparing mouse IgG immunosensor on the electrode with GC and poly(*o*-ABA), optimization could be achieved without being treated previously and being previously treated using a current of 25  $\mu\text{A}$  lasting for 300 s, as well as curing temperatures up to 200  $^{\circ}\text{C}$ , 250  $^{\circ}\text{C}$ , and 300  $^{\circ}\text{C}$  to prepare GC electrode prior to poly(*o*-ABA) electrical polymerization. The optimised curing temperatures have been obtained through 1 mM  $\text{K}_4\text{Fe}(\text{CN})_6$ 's maximum current response, with its highest response monitored on that of GC. The curing temperature for it was 200  $^{\circ}\text{C}$  after it was treated

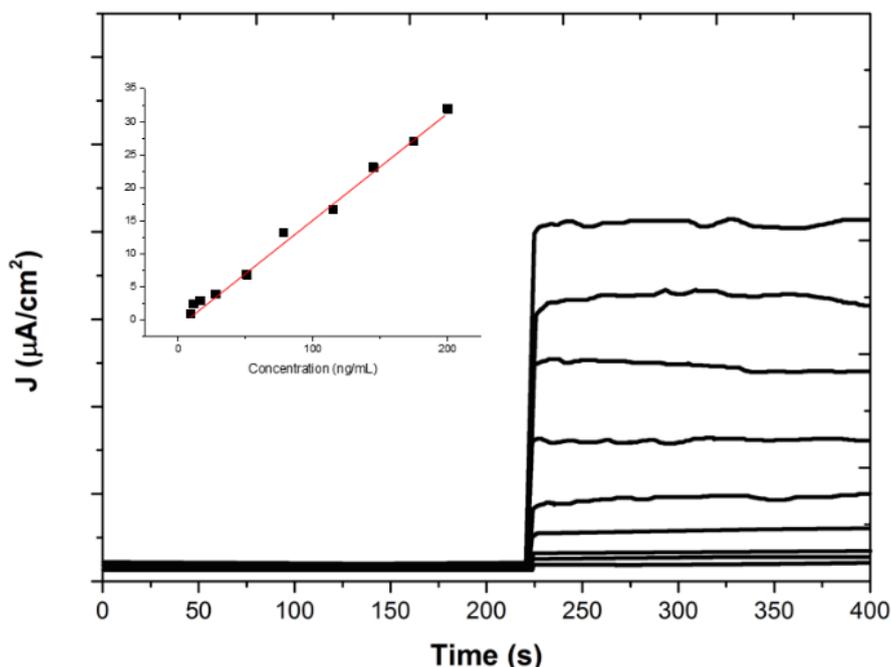
previously with an anodic current of 25  $\mu\text{A}$  lasting 300 s. The best pre-treatment of GC electrode went through measurement by immunoassay with the target's maximum proportion to be 1000 ng/mL mouse IgG to control as 0 ng/mL mouse IgG. Having been treated previously, the GC electrode appearance possesses hydrophilic trait, thus facilitating the electropolymerization process compared with a GC electrode without being treated previously.

Fig. 5A–C indicates the amperometric responses of AA on mouse IgG immunosensors. AA was produced through ALP enzyme. With the potentials of 0.40 V, 0.40 V, and 0.50 V, the experiment was conducted on the basis of electrodes of poly(*o*-ABA) together with GC, Au, as well as SPC correspondingly. On every electrode immunosensor, the fitful potentials could be gained if measured through hydrodynamic voltametric. The current density of target were 22.49  $\mu\text{A}/\text{cm}^2$ , 33.10  $\mu\text{A}/\text{cm}^2$  and 43.50  $\mu\text{A}/\text{cm}^2$  for poly(*o*-ABA) together with SPC, GC as well as Au correspondingly. Through the analysis of the experiment, the maximum response ratio of current density between control group and target group, roughly 297, was achieved with mouse IgG immunosensor on poly(*o*-ABA)/Au. This suggested the poly(*o*-ABA) on the appearance of Au were likely to generate an appearance which has the minimum no-certain adherence. Therefore, in the following research, such immunosensor was preferably adopted as to mouse IgG. The use of typical ensemble properties of nanoparticles as a recognition signal resulted in the detection limit for the target IgG of about 100 pM. The target IgG at the 10 pM level, however, was identified utilizing the electrocatalytic amplification of single nanoparticle collisions [34]. For the behavior of larger molecule of substrate may exhibit slower turnover in the enzymes reaction and may also exhibit slower diffusion though the bilayer towards the electrode surface. Under such circumstances, using small molecules of AAP, after the AAP substrate is added in the Tris buffer solution, it can be generated to AA very fast, becoming a stable current within 30 s at all of three immunosensors. Preparing mouse IgG SAM/Au immunosensors achieved optimization 6-mercapto-1-hexanol with diverse proportions was obtained from 11-mercaptoundecanoic acid. The adoption of 1:9 to be single layer mixture contributed to the generation of the maximum proportion for target to control, probably since the elected proportion of 6-mercapto-1-hexanol could achieve the minimization of non-certain adsorption in an efficient way. Meanwhile, adequate binding could be supplied by 11-mercaptoundecanoic acid to anti-mouse IgG.



**Figure 5.** Amperometric responses of 1000 ng/mL and 0 ng/mL mouse IgG at (A) poly(*o*-ABA)/GC, (B) poly(*o*-ABA)/Au and (D) SAM/Au immunosensors in 0.5 M Tris buffer solution.

The poly(*o*-ABA)/Au immunosensing system responds with its best sensitivity to detecting mouse IgG. As indicated in Fig. 6, diverse mouse IgG concentrations' amperograms on the relationship between time and current density response. Graphic plots of the initial rate of hydrolysis versus the enzyme concentrations, expressed in mg/ml of total solution, yielded straight lines for all three enzyme-substrate reactions [35]. They have been measured triply with diverse immunosensors. The figure indicated the ever-changing range of the relationship between mouse IgG concentration and the mean performance of current density responses. As is shown, the dynamic ranges from 2 ng/mL to 400 ng/mL. Meanwhile, the boundary figure for detection is 0.7 ng/mL. The sensitivity of the poly(*o*-ABA)/Au was compared with that of other reported modified electrodes and the results were presented in Table 1.



**Figure 6.** Amperograms of different concentration of mouse IgG at poly(*o*-ABA)/Au immunosensor in 0.5 M Tris buffer solution (pH8.5).

**Table 1.** Comparison of the present poly(*o*-ABA)/Au with other IgG determination reports.

Electrode	Linear detection range	Detection limit	Reference
Ag (I)-cysteamine complex	0.1-20 ng/mL	0.4 fg/mL	[36]
PTH-MB/GNP/antibody electrode	10-4000 ng/mL	3 ng/mL	[37]
Poly(pyrrole-3-carboxylic acid)	10-440 ng/mL	1 ng/mL	[38]
poly( <i>o</i> -ABA)/Au	2-400 ng/mL	0.7 pg/mL	This work

#### 4. CONCLUSIONS

With its sound performance in sensitivity and diverse adoptable substrates, alkaline phosphatase is regarded to be a good choice for label in the field of electrochemical immunoassays. Through CV as well as amperometry, ALP substrates 7 resultants went through examination and comparison. as a result, AAP substrate's AA product generated a signal that is strong and steady. Besides, the needed substrate costs comparatively less. Thus it could be regarded as a good choice for immunosensors at GC and Au electrodes.

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