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Poly(noradrenalin) based bi-enzyme biosensor for ultrasensitive multi-analyte determination

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ABSTRACT

In order to realize the multi-analyte assays for physiological molecules and environmental contaminants, a bi-enzyme biosensor based on horseradish peroxidase (HRP)-catalyzed noradrenalin (NA) polymerization in the presence of H_2O_2 was fabricated for the first time and utilized in immobilizing HRP and glucose oxidase (GOx) simultaneously. The resultant bi-enzyme modified electrode was demonstrated to be efficient in monitoring multi-analyte (H_2O_2, Cr(III), glucose, and Cr(VI)). It was shown that the prepared PNA-HRP-GOx/Pt electrode exhibits a sensitivity (*S*) high up to 628.4 μA mM^{-1} cm^{-2} in the linear range (LR) of 0.50 μM ~ 0.42 mM and a *S* of 208.9 μA mM^{-1} cm^{-2} in the LR of 0.42 mM~3.5 mM in glucose sensing, with a limit of detection (LOD) of 0.08 μM observed; in Cr(VI) sensing a LOD of 0.20 nM and a LR of 0.50 ~ 6.0 nM were obtained. With the addition of polyaniline (PANI), the resultant PNA-HRP-GOx/PANI/Pt electrode potentiostated at -0.20 V responded linearly to H_2O_2 concentration in 0.05 ~ 30.2 mM range, with linearly responses to Cr(III) concentration in the LR of 0.01 ~ 3.8 μM. Hence, an amperometric biosensor with high *S*, low LOD and good anti-interference ability for detection of glucose, H_2O_2 and heavy metal ions (Cr(III) and Cr(VI)) was developed.

Graphical abstract

fx1

Keywords: oxidative polymerization of norepinephrine, biosensing, glucose, H_2O_2, Cr(III),

Cr(VI).

1. Introduction

The requirement for sensitive techniques capable of multi-analyte detection in a single sample has become extremely critical in the fields of environment, medical care, food, anti-terrorism etc. over the past few decades [1-2]. Discriminative detection of multiple analytes is very challenging and commonly requires bulky and sophisticated instrumentations [3-4]. Biochemical or chemical sensors serve well in multi-analyte determination and do not need complicated and time-consuming pre-concentration procedures, expensive testing cost, and large-scale analytical instruments, so they are expected to have wide applications such as in-field and on-line testing [5]. Therefore many research focus on multi-analyte sensors development and addressing problems inherent in discriminating multiple simultaneous signals without loss in measurement speed, specificity or sensitivity [6-7].

H_2O_2 plays a crucial role in various physiological processes and serves as a bridge in the assays of many enzymes and their products. Therefore, the detection of H_2O_2 is of great significance both in enzyme-based assays and in metabolism control because of its biological role as messenger molecule in living bodies [8-10]. The high demand for blood glucose monitoring intrigues significant research and development efforts devoted to developing reliable glucose sensors for in vitro or in vivo applications [11-15]. Heavy metals (HMs), which are non-biodegradable and can accumulate in human and animal tissues, are one of the main causes of serious pollution in the world which can lead to inhibition in metabolism and in the function of numerous enzymes and hormones [16-18]. Chromium (Cr) is a common contaminant in surface water and groundwater resulting from numerous industrial and military activities [19]. Cr(VI) is non-degradable and has mutagenicity and carcinogenicity [20]. Its toxicity is considered to be 500 to 1000 times that of Cr(III) because of its strong oxidizing properties, as water-soluble oxygen anions on plants, animals and humans have serious harm, with Cr(VI) concentration limited to be a few ppb in groundwater and potable water supplies [21-22]. The determination and control of Cr(III) is also very important

because Cr(III) and Cr(VI) can be transformed into each other and Cr(III) also have great impacts on health at high or at very low concentrations [23-24]. Due to the widespread and the high toxicity of HMs (Cr(III) and Cr(VI)), it is important to develop sensitive and selective identification techniques to detect them in environmental monitoring [25-26].

With the rapid development of biology, chemistry, physics, medicine, and electronic technology over the past few decades, enzyme-based biosensing is one of the most active research areas in analytical chemistry due to its incomparable advantages including high sensitivity and high selectivity over other techniques and have been widely applied to diverse biomedical, environmental and food safety applications [27]. The co-immobilization of multi-enzymes on an electrode is expected to serve well in multiple analytes detection because enzymes can be used for selectively catalytic sensing of important substrates and inhibitive assays of various environmental pollutants [6, 28]. An excellent multienzymatic-rotating biosensor fabricated with the combination of cholesterol esterase, cholesterol oxidase and peroxidase was reported and applied for total cholesterol determination [29], which exhibiting good promise in biological sensing. Among various matrices utilized in enzyme immobilization, noradrenalin polymer (PNA) was demonstrated to be of great performance in constructing enzyme-based biosensors [30]. In our previous work, the electro-synthesized PNA matrix was found to be of the lowest competitive affinity to HMs and exhibited the highest sensitivity for the inhibitive assay of HMs as compared with several other polymer matrices [31]. However, as known to us, the enzyme-catalyzed polymerization of NA and its utilization in entrapping bi-enzyme molecules for multi-analyte monitoring has never been reported, which is expected to be meaningful and promising.

Herein, we report on the HRP-catalyzed polymerization of NA in the presence of H_2O_2 as a new and efficient platform to immobilize HRP and GOx simultaneously in polymeric bionanocomposite cast thin films. The thus-prepared biosensors were demonstrated to be of excellent performance in sensitively and selectively determining multiple analytes including glucose, H_2O_2, Cr(III) and Cr(VI), at the same time exhibiting high sensitivity, rapid response, good selectivity and stability without the need for outer-layer fixed film.

2. Experimental

2.1. Instrumentation and Chemicals

All electrochemical experiments were conducted on a CHI660C electrochemical workstation (CH Instrument Co.). The Pt electrode of 3.0 mm diameter served as the working electrode, a KCl saturated calomel electrode (SCE) as the reference electrode, and a carbon rod as the counter electrode. All potentials are cited versus SCE. Polyaniline (PANI) modified Pt electrode (PANI/Pt) was prepared by continuously scanning the potential between 0.20 V and 1.10 V at 50 mV s^{-1} in 1.0 M HCl solution containing 0.20 M aniline. Scanning electron microscopy (SEM) studies were performed on a Hitachi S4800 scanning electron microscope with a field emission electron gun.

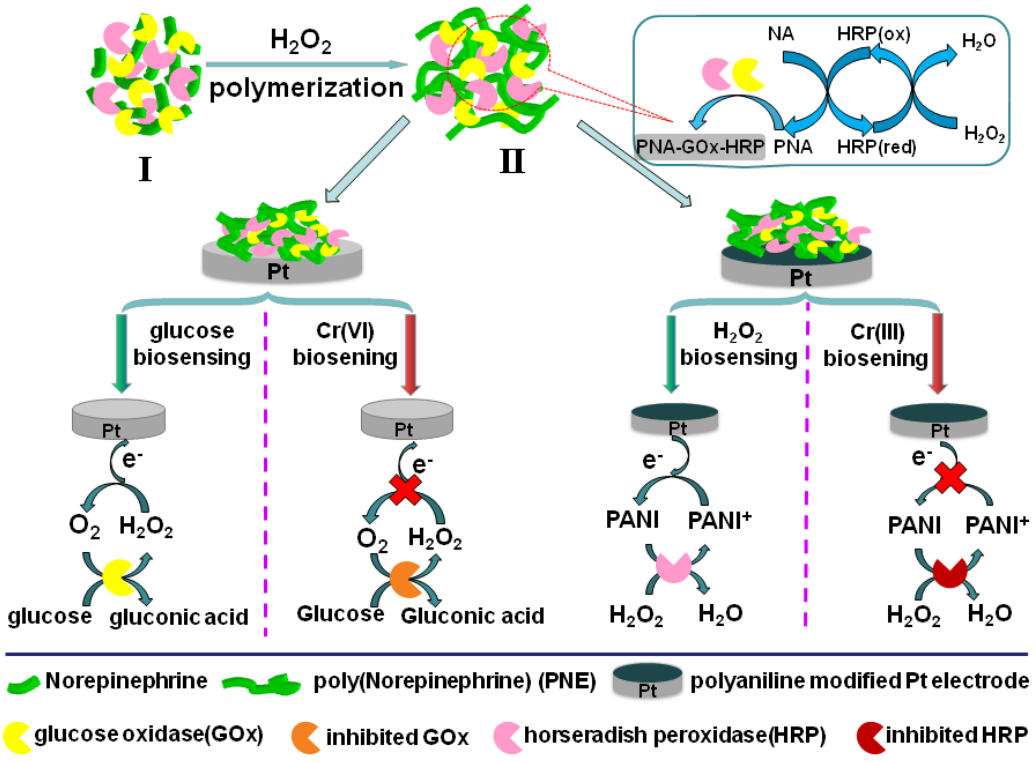
HRP (EC.1.11.1.7; 400 U mg^{-1}), GOx (EC 1.1.3.4; type II from Aspergillus niger, 150 U mg^{-1}), and norepinephrine were obtained from Sigma-Aldrich. Glucose, $Cr(NO_3)_3{\cdot}9H_2O$ and $K_2Cr_2O_7$ were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 30 wt% H_2O_2 solution was purchased from Shanghai Taopu Chemical Factory and diluted daily before use. 1.0 M glucose stock solution was allowed to mutarotate overnight at room temperature before use. Cr(III) and Cr(VI) solution were daily prepared from $Cr(NO_3)_3{\cdot}9H_2O$ or $K_2Cr_2O_7$, respectively. Other reagents were of analytical grade or better quality and used as received. Milli-Q ultrapure water (Millipore, ≥18 MΩ cm) was used throughout. All experiments were performed at room temperature around 25 ^oC.

2.2. Procedures

As shown in Scheme 1, a PBS (30 mM, pH 7.0) solution containg 2.5 mM NA + 4.0 mg mL^{-1} HRP + 4.0 mg mL^{-1} GOx was prepared. With the addition of 10 mM H_2O_2 (final concentration), NA would polymerize very fast under HRP-catalyzed oxidation and the resultant NA polymer (PNA) entrapped HRP and GOx at the same time [32].

The prepared bi-enzyme-entrapped biocomposite (5 μL) was then cast on bare Pt electrode or PANI/Pt electrode and incubated at 37 ^oC for 1 h in a water bath and taken out till air-dried. The prepared PNA-HRP-GOx/Pt electrode was used for glucose and Cr(VI) biosensing and the PNA-HRP-GOx/PANI/Pt electrode was used for H_2O_2 and Cr(III) sensing. The conditions for fabricating PNA-HRP-GOx/Pt electrode and for enzyme substrates or enzyme inhibitors

biosensing had all been carefully examined and optimized experimentally. And the sensing performance of the resultant biosensors were all examined under optimized conditions in the following experiments. In the glucose biosensing, the PNA-HRP-GOx/Pt electrode was immersed in 30 mM PBS (pH 7.0) and potential-stated at 0.60 V. While in the inhibitive sensing of Cr(VI), the PNA-HRP-GOx/Pt electrode was immersed in acetate buffer solution (pH 3.6) and the inhibition percentage (In(%)) aroused by the inhibitor Cr(VI) was evaluated according to the steady-state current response (I_0) with the addition of 0.05 mM glucose (substrate) and the corresponding current after the addition of certain concentrations of inhibitor (I_i). The inhibition percentage (In(%)) aroused by the inhibitor Cr(VI) was evaluated according to the equation: In(%) = (I_0 - I_i)/I_0, which was proportional to the final concentration of inhibitor in solution. The determination of H_2O_2 with PNA-HRP-GOx/PANI/Pt electrode was also carried out in 30 mM PBS (pH 7.0) and -0.20 V was chosen as the analytical potential. And Cr(III) sensing was also carried out with PNA-HRP-GOx/PANI/Pt electrode based on the inhibitive mode, pH 5.0 PBS (30 mM) was chosen for Cr(III) sensing and the analytical potential was optimized as -0.20 V.



Scheme1. Schematic representation of procedures for preparing PNA-HRP-GOx and its applications in detection of glucose/H_2O_2 and inhibitive-based detection of Cr(III)/Cr(VI).

3. Results and Discussion

3.1. Enzyme-Catalyzed Polymerization of NA. According to the literature, HRP has been widely used for oxidative polymerization of phenolic compounds in the presence of H_2O_2 [33]. For the first time, HRP accompanying with H_2O_2 was utilized here to catalyze the polymerization of NA in aqueous solution. As shown in Figure 1, for 2.5 mM NA (1), 2.5 mM NA + 10 mM H_2O_2 (2), and 2.5 mM NA + 4.0 mg mL^{-1} HRP (3), the solution was colorless and changed little even after 5 h; while the color of solutions of 2.5 mM NA + 4.0 mg mL^{-1} GOx (4), 2.5 mM NA + 4.0 mg mL^{-1} GOx + 10 mM H_2O_2 (5), and 2.5 mM NA + 4.0 mg mL^{-1} HRP + 4.0 mg mL^{-1} GOx (6) was light yellow due to the existence of GOx, which also remained the same after 5 h. As shown in solution (7) and (8), as soon as 10 mM H_2O_2 (final concentration) was added into 2.5 mM NA + 4.0 mg mL^{-1} HRP (3) or 2.5 mM NA + 4.0 mg mL^{-1} HRP + 4.0 mg mL^{-1} GOx (6), the solutions turned brown instantly, indicating the immediate onset of NA oxidation catalyzed by HRP and H_2O_2. The solution turned black after quiescence for 30 min, and some precipitates were observed at the bottom of the centrifuge tube. Comparing these 8 kinds of solutions, it was easy to conclude that the oxidation and polymerization of NA occurred very slowly here in the absence of HRP or H_2O_2, while with the co-existence of HRP and H_2O_2 in solution, the polymerization of NA could carry out very fast. This demonstrated that NA can polymerize fast under the function of the product of HRP-catalyzed H_2O_2 oxidation (the oxidation state of HRP), as demonstrated in the insert of Scheme 1. And the resultant PNA was expected to form bi-enzyme-entrapped biocomposites with the presence of HRP and GOx, which was demonstrated in the following experiments.

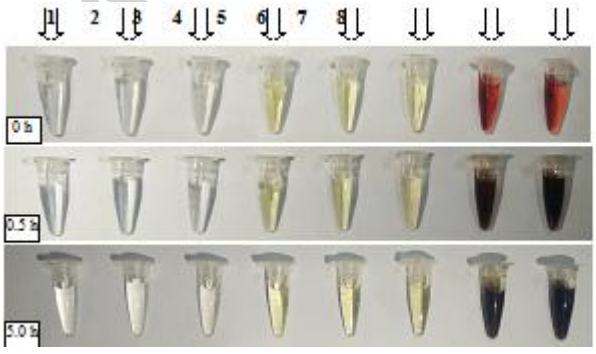


Fig. 1. Digital pictures of various 30 mM PBS suspensions containing (1) 2.5 mM NA; (2) 2.5 mM NA + 10 mM H_2O_2; (3) 2.5 mM NA + 4.0 mg mL^{-1} HRP; (4) 2.5 mM NA + 4.0 mg mL^{-1} GOx; (5) 2.5 mM

NA + 4.0 mg mL^{-1} GOx + 10 mM H_2O_2; (6) 2.5 mM NA + 4.0 mg mL^{-1} HRP + 4.0 mg mL^{-1} GOx; (7) 2.5 mM NA + 4.0 mg mL^{-1} HRP + 10 mM H_2O_2; (8) 2.5 mM NA + 4.0 mg mL^{-1} HRP + 4.0 mg mL^{-1} GOx + 10 mM H_2O_2 after 0 h (panel 1), 0.5 h (panel 2), and 5.0 h (panel 3).

As shown in Figure 2, the FTIR spectra of NA (a), PANI (b), PNA-HRP-GOx (c) and PNA-HRP-GOx/PANI (d) were examined for comparison. The significant bands of 3500 ~ 3200 cm^{-1} in the spectra of NA should be assigned to stretching or deformation vibrations of phenolic O–H bonds; and the long bands of 1500 ~ 600 cm^{-1} in NA spectra can be attributed to significant bands of the phenolic O–H bonds, which contain the surface deformation vibration peak and the out-of-plane deformation vibration peak of O–H bonds (spectrum a); typical PANI spectra were observed in spectrum b; bands around 1650 cm^{-1} in PNA can be attributed to the stretching of aromatic C–C bonds and the C=O in newly formed benzoquinone (spectrum c); bands from 950 to 650 cm^{-1} are attributed to out-of-plane deformation of aromatic C–H bonds [34]. It is shown that the peaks at 1560 cm^{-1} and 1470 cm^{-1} correspond to C-C stretching modes of quinoid and benzenoid rings in PANI. The bands at 1298, 1161 and 825 cm^{-1} in spectra d is in corresponding to vibrations of C-N bonds in the quinoid and benzenoid rings and to C-H bending in plane or out of plane, respectively [35]. This demonstrated that NA was effectively oxidized by HRP and the PANI film was successfully obtained by electrochemical polymerization.

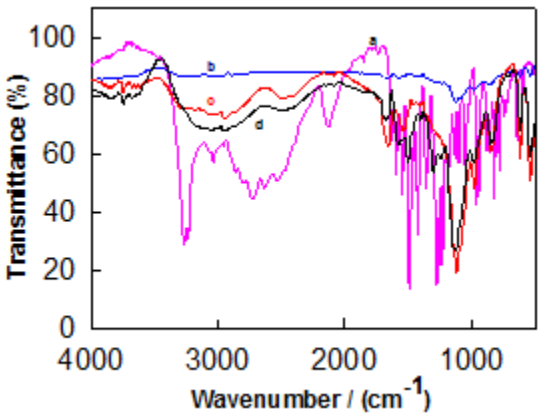


Fig. 2. FT-IR spectra of NA (a, pink), PANI (b, blue), PNA-HRP-GOx (c, red), and PNA-HRP-GOx/PANI (d, black).

The SEM images of PNA-HRP-GOx (A), PANI (B) and PNA-HRP-GOx/PANI film (C) were collected and shown in Figure 3. With the coating of PNA-HRP-GOx (A), we can clearly observe a uniform film covered on the surface of electrode, demonstrating that the

enzyme-catalyzed NA polymerization was very gentle and the resultant bienzyme-entrapped biocomposites were thus uniformly distributed. The electrically polymerized PANI exhibited a crosslinked nanowire network in Figure 3(B). And the film of PNA-HRP-GOx/PANI shown in Figure 3(C) exhibits a porous structure similar to the PANI film, with some additional films formed on the PANI network, demonstrating the resultant film was also very uniform and the porous structure is suitable for mass transfer and beneficial for biosensing applications.

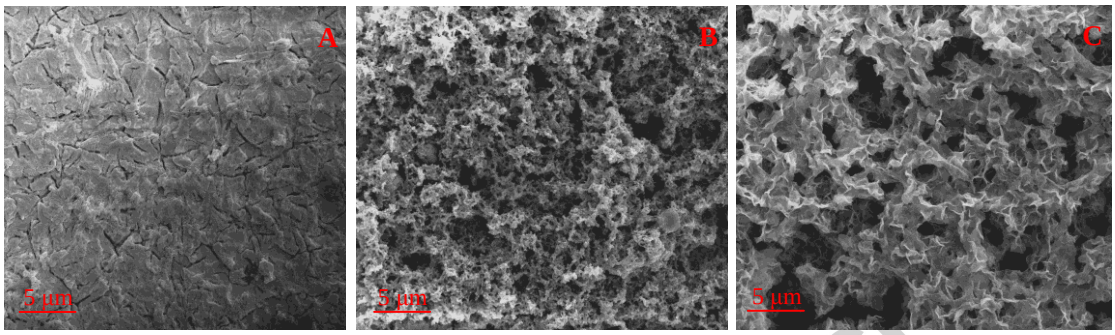


Fig. 3. SEM images of the PNA-HRP-GOx (A), PANI (B) and PNA-HRP-GOx/PANI (C).

3.2. Analytical Performance of the PNA-HRP-GOx biocomposites

3.2.2. Performance of PNA-HRP-GOx/Pt for the determination of glucose and Cr(VI)

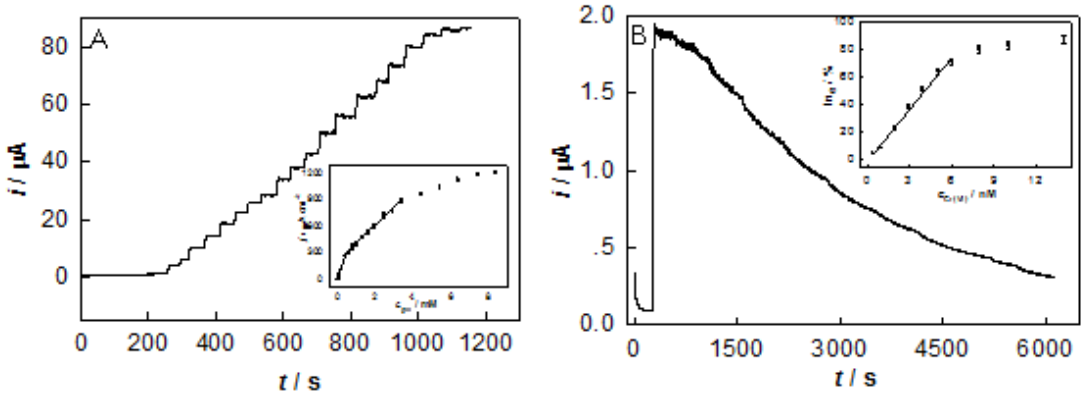


Fig. 4. Time-dependent current responses (A) to successive additions of glucose into stirred 30 mM PBS (pH 7.0) and the calibration curve at the PNA-HRP-GOx/Pt electrode (insert). (B) The inhibition current responses curves of the PNA-HRP-GOx/Pt electrode to continuous additions of different concentrations of Cr(VI) prepared in acetate buffer solution (pH 3.6) and the calibration curve for Cr(VI). Applied potential: +0.60 V vs. SCE.

PNA-HRP-GOx/Pt electrode was prepared and utilized for the analysis of the substrate (glucose) and the inhibitor (Cr(VI)) of GOx enzymatic reaction. Figure 4(A) shows the typical steady state chronoamperometric response of the PNA-HRP-GOx/Pt potential-stated at 0.60 V to the successive additions of glucose into stirred 30 mM PBS (pH 7.0). The

biosensor displays excellent performance in glucose biosensing, with high sensitivity and very low LOD (80 nM glucose) observed. Figure 4(B) illustrates the time-dependent response of the PNA-HRP-GOx/Pt to additions of Cr(VI). In Cr(VI) biosensing, the prepared PNA-HRP-GOx/Pt potential-stated at 0.60 V was immersed in acetate buffer solution (pH 3.6), 0.05 mM of glucose (substrate) was added and a steady-state current response (I_0) was reached. Then, the concentration of the added Cr(VI) increased stepwisely, with the corresponding current after adding certain concentrations of inhibitor (I_i) recorded. The inhibition percentage (In(%)) aroused by the inhibitor Cr(VI) was evaluated according to the equation: In(%) = $(I_0 - I_i)/I_0$, which was proportional to the final concentration of inhibitor in solution. As shown in Figure 4(B) and list in Table 1, PNA-HRP-GOx/Pt electrode can detect as low as 0.2 nM Cr(VI). The biosensing performance of the prepared biosensor to glucose and Cr(VI) including the corresponding sensitivity (S), limit of detection (LOD), and linear range (LR) is presented in Table 1. We can clearly see the biosensors proposed in our novel method exhibits high sensitivity, low LOD and relative wide LR in glucose and Cr(VI) sensing by comparing with most reported values. It is worth to point out that the conditions for enzyme substrates or enzyme inhibitors biosensing had all been carefully examined and optimized by experiment, and the sensing of glucose carried out in pH 7.0 PBS and the inhibitive sensing of Cr(VI) carried out in pH 3.6 acetate buffer solution. That may because the neutral medium of pH 7.0 PBS is beneficial for GOx activity, while Cr(VI) exhibiting the highest inhibitive effect in the acidic medium of pH 3.6 acetate buffer solution. As shown in Table 1, the PNA-HRP-GOx/Pt electrode exhibited excellent performance in glucose and Cr(VI) biosensing comparing with literature values, demonstrating the great advantages of the resultant biosensor.

3.2.2. Performance of PNA-HRP-GOx/PANI/Pt for the determination of H_2O_2 and Cr(III)

We also examined the performance of the resultant PNA-HRP-GOx/PANI/Pt electrode in H_2O_2 and Cr(III) biosensing. Figure 5(A) shows the steady-state current response of the PNA-GOx-HRP/PANI/Pt electrode to successive additions of H_2O_2 in 30 mM PBS (pH 7.0) at -0.20 V, with the corresponding calibration curve for H_2O_2 shown in the insert of Figure 5(A). The PNA-HRP-GOx/PANI/Pt electrode can also be used in chronoamperometric

determination of Cr(III) because of the inhibition effect of Cr(III) on HRP activity. Figure 5(B) presented the time-dependent response of the PNA-GOx-HRP/PANI/Pt electrode to continuous additions of Cr(III) in 30 mM PBS (pH 5.0) at -0.20 V. The performance of the prepared biosensors in H_2O_2 and Cr(III) sensing (includes the *S*, LOD, and LR) were presented in Table 1. As comparing with many reported values, our PNA-HRP-GOx/PANI/Pt electrode exhibits high sensitivity and low LOD in H_2O_2 and Cr(III) sensing. The biosensors prepared in this paper are also the first successful example of realizing four kinds of analytes biosensing with a single biocomposites. These results indicate that the novel PNA-HRP-GOx serves well as an excellent biosensing platform, which can detect a variety of substances.

Table 1. Comparison of performances of proposed biosensors for glucose, Cr(VI), H_2O_2, and Cr(III) with reported values.

Immobilization method	Detection substrate	LOD (μM)	Sensitivity (μA mM^{-1})	Linear Range (μM)
PD-GOx-Tyr/Pt[36]	glucose	0.1	5.55	2.0 ~ 5.7×10^3
PtPd-MWCNTs-GOx/GCE[37]	glucose	0.031	7.91	6.2 ~ 1.4×10^4
Pt-N-GSS-GOx/GCE[38]	glucose	1	6.36	10 ~ 1.255×10^4
*This method	glucose	0.08	44.39 14.75	0.5~ 418 418~3.42×10^3
$GOx/SPC_{Pt}E$[39]	Cr(VI)	9.0×10^{-2}	/	9.0×10^{-2} ~ 0.796
HRP/CFE[40]	Cr(VI)	9.0×10^{-2}	/	9.0×10^{-2} ~ 1.5
*This method	Cr(VI)	2.0×10^{-4}	/	5.0×10^{-4} ~ 6.0×10^{-3}
PANI/HRP/Pt[41]	H_2O_2	0.04	1.75	50~ 3.17×10^3
PANI-co-PDTDA/HRP[42]	H_2O_2	0.859	25.24	5~34
PANI/HRP/ SPCEs[43]	H_2O_2	32	/	43~750
HRP/PANI/CS[44]	H_2O_2	0.5	/	10~1.5×10^3
∗This method	H_2O_2	10	2.40	50 ~ 3.02×10^4
Tyr/Ppy[45]	Cr(III)	0.5	/	0.5~ 5.0
HRP/PNR/CFE[40]	Cr(III)	0.27	/	0.27 ~ 5.1
∗This method	Cr(III)	0.01	/	0.01 ~ 3.8

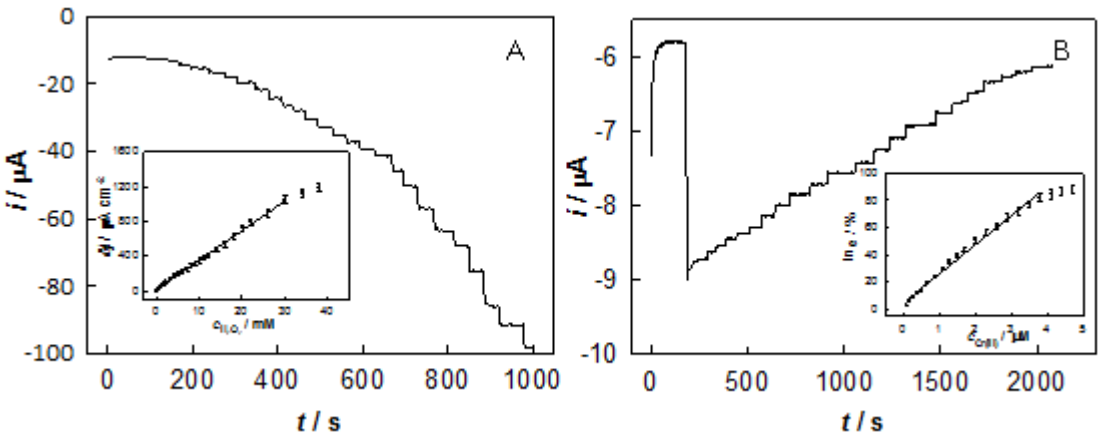


Fig. 5. Time-dependent current responses of PNA-HRP-GOx/PANI/Pt electrode to successive additions of H_2O_2 into stirred 30 mM PBS (pH 7.0) and the corresponding calibration curve (A). The inhibition current responses of PNA-HRP-GOx/PANI/Pt electrode to continuous additions of different concentration of Cr(III) solution in 30 mM PBS (pH 5.0) with the calibration curve for Cr(III) shown as the insert (B). Applied potential: -0.20 V vs. SCE

3.2.3. Selectivity and stability of the prepared biosensors

Selectivity is an important parameter in the performance of a biosensor. In order to confirm the selectivity of the prepared biosensor, we studied the common interferences in glucose, Cr(VI) and H_2O_2 biosensing. As shown in Figure 6(A), for the measurement of 0.15 mM glucose, 100 mM NaCl, 1.0 mM galactose, 0.10 mM acetaminophen, 0.05 mM uric acid (UA), 0.05 mM ascorbic acid (AA), 100 mM KCl, and 1.0 mM sodium acetate have no obvious interference. The responses of PNA-HRP-GOx/Pt electrode to the sequential additions of 3.0 nM Cr(VI) solution, 20 μM Cu^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Fe^{3+}, Hg^{2+}, Pb^{2+}, Cr^{3+}, As^{3+}, Ag^{+} and 3.0 nM Cr(VI) solution into acetate buffer solution (pH 3.6) was shown in Fig. 6(B), which demonstrated the excellent anti-interference ability of the resultant PNA-GOx-HRP/Pt electrode, and have potential applications in the determination of Cr(VI) in real water samples. As shown in Figure 6(C), for the measurement of H_2O_2, 0.10 mM glucose, 0.20 mM uric acid (UA) and 0.20 mM ascorbic acid (AA) have little response on PNA-HRP-GOx/PANI/Pt electrode.

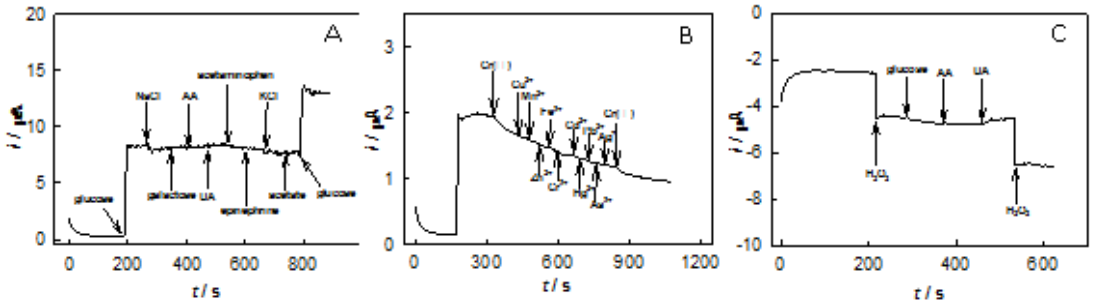


Fig. 6. (A) Effect of additions of 0.20 mM glucose, 100 mM NaCl, 1.0 mM galactose, 0.05 mM ascorbic acid (AA), 0.05 mM uric acid (UA), 0.1 mM acetaminophen, 100 mM KCl, 1.0 mM sodium acetate, and 0.20 mM glucose on the responses of PNA-HRP-GOx/Pt electrode. (B) Current responses at the PNA-HRP-GOx/Pt electrode to the sequential additions of 0.05 Mm glucose, 3.0 nM Cr(VI), 20 μM Cu^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Fe^{3+}, Hg^{2+}, Pb^{2+}, Cr^{3+}, As^{3+}, Ag^{+} and 3.0 nM Cr(VI) solution into acetate buffer solution (pH 3.6). The measurements were conducted at +0.60 V vs.SCE. (C) Effect of additions of 0.5 mM H_2O_2, 0.10 mM glucose, 0.20 mM AA and 0.20 mM UA on the response of the the PNA-HRP-GOx/PANI/Pt electrode.

When not in use, the prepared GOx electrodes were stored in pH 7.0 PBS at 4 ^{o}C (refrigerator). The long term stability of the prepared biosensor to multi-analytes sensing was evaluated by monitoring the amperometric responses to additions of 1.0 mM glucose or 0.50 mM H_2O_2 in 30 mM PBS (pH 7.0), or to 3.0 nM Cr(VI) in acetate buffer solution (pH 3.6), or to 1.0 μM Cr(III) in 30 mM PBS (pH 5.0) at a period of 6 weeks. The responses of the optimized biosensor to glucose and H_2O_2 can maintain over 80% of the initial values after storing in 30 mM PBS (pH 7.0) for 30 days. It was also demonstrated experimentally that the prepared biosensor can be used repeatedly in the determination of Cr(VI) and Cr(III) for 6 weeks, and a decrease of 40% from the initial response was observed after 6 weeks. This demonstrated that the prepared biosensor had excellent long stability.

The intra- and inter-day precision (coefficient of variation, CV(%)) and accuracy (bias(%)) of the biosensors proposed in this paper in glucose and H_2O_2 determination were determined by analyzing 5 samples at different concentrations in the same day and one sample at each substrate concentration in five different days, respectively (Table 2) [29]. As shown in the table, the proposed biosensors exhibited excellent intra- and inter-day precision and accuracy, and the percentage standard error was less than 4%. This can be explained as the enzymatic-synthesized PNA was an excellent matrix in immobilizing enzymes and the resultant biosensors had excellent stability and anti-interference ability, so excellent precision and accuracy was obtained here.

Table 2. Intra- and inter-day precision and accuracy in glucose and H_2O_2 biosensing (n = 5)

	Intra-day						Inter-day					
	glucose			H_2O_2			glucose			H_2O_2		
$c_{nominal}$ (mM)	3.92	5.87	8.65	0.145	0.586	1.47	3.92	5.87	8.65	0.145	0.586	1.47
c_{found} (mM)	3.68	5.94	8.36	0.136	0.565	1.35	3.56	5.74	8.25	0.131	0.573	1.33
CV (%)	1.60	1.42	2.18	1.48	1.67	2.31	1.26	1.89	3.42	1.52	1.74	2.98
Bias (%)	6.12	1.19	3.35	6.21	3.58	8.16	9.18	2.21	4.62	9.66	2.22	9.52

3.2.4. Real sample determination with the prepared biosensors

To testify the feasibility of the developed sensor for practical applications, the biosensors developed in this paper were used to detect different substrates in real samples by using standard addition method. The real samples were pretreated according to the literatures and the sample solutions were prepared with the conditions identical to the sensing experiments mentioned above. The applicability of the PNA-HRP-GOx-based biosensors for glucose determination in real samples was carried out by determining glucose in human serum samples. H_2O_2 determination in real samples was realized by analyzing H_2O_2 in ready-made milk. The ability of the developed biosensors to detect Cr was tested in water samples collected from a chromite mine. It can be observed from Table 3 that the biosensors could detect glucose, H_2O_2, Cr(VI), and Cr(III) present in real samples with RSD in the range from 2% to 6% and the recovery in the range from 91.0% to 114.0%. The result implied that the PNA-HRP-GOx-based biosensors proposed in this paper had great prospects in real sample analysis.

Table 3. The Recovery of glucose, H_2O_2, Cr(VI), and Cr(III) in real samples tested with PNA-HRP-GOx-based biosensors.

Sample		Added (mM)	Found (n = 3, mM)	Recovery (%)	RSD[a](%)
glucose	I	1.0	0.94	94.0	2.7
	II	1.5	1.46	97.3	3.4
	III	2.0	2.12	106.0	1.8
H_2O_2	I	1.0	0.92	92.0	3.7
	II	2.0	1.82	91.0	4.2
	III	5.0	5.17	103.4	3.6
Cr(VI)	I	1.0×10^{-6}	1.14×10^{-6}	114.0	4.6
	II	2.0×10^{-6}	1.86×10^{-6}	93.0	4.5
	III	3.0×10^{-6}	2.80×10^{-6}	93.3	3.8
Cr(III)	I	5.0×10^{-4}	5.06×10^{-4}	101.2	4.2
	II	1.0×10^{-3}	1.05×10^{-3}	105.0	4.6
	III	1.5×10^{-3}	1.69×10^{-3}	112.7	5.3

[a]Relative standard deviation (RSD) of 3 measurements.

4. Conclusions

In summary, we have successfully synthesized PNA-HRP-GOx composite with NA as the monomer and HRP as the catalyst to realize the polymerization of NA and the co-immobilization of GOx and HRP in the presence of H_2O_2. The fabrication of the biosensor can be finished in 1.5 h and the total assay time of the method developed is within 20 min. For the first time, this report realized the analysis of glucose, H_2O_2 and heavy metal ions (Cr(III) and Cr(VI)) with a single biosensor. The thus-prepared bi-enzyme biosensor exhibits high sensitivity, low LOD, and good selectivity for the detection of four kinds of analytes. Compared with conventional biosensors, the advantages of the sensors prepared in this paper include the ability to be able to reduce the cost and improve the efficiency in multi-analyte sensing applications. They are expected to be widely utilized in real complex sample analysis. This technique can also find wider applications in fabricating excellent multi-enzyme biosensing platform and can be utilized in immobilizing various biomolecules, which is expected to be of great prospects in biosensing areas.

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Highlights

(1) Effective co-immobilization of Horseradish peroxidase (HRP) and glucose oxidase (GOx) via HRP-catalyzed polymerization of noradrenalin in the presence of H_2O_2.

(2) The thus-prepared HRP and GOx immobilized electrodes exhibit excellent performance superior to most previous reported values.

(3) The proposed biosensors realized the ultrasensitive biosensing of multi-analyte (including H_2O_2, glucose, Cr(III), and Cr(VI)).