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A Stable Sandwich-Type Hydrogen Peroxide Sensor Based on Immobilizing Horseradish Peroxidase to a Silver Nanoparticle Monolayer Supported by PEDOT:PSS-Nafion Composite Electrode

Yuanyuan Yao[1], *Yangping Wen*[1,2], *Long Zhang*[1], *Jingkun Xu*[1,*], *Zifei Wang*[1], *Xuemin Duan*[3]

[1]Jiangxi Key Laboratory of Organic Chemistry, Jiangxi Science and Technology Normal University, Nanchang 330013, P. R. China
[2]Key Laboratory of Crop Physiology, Ecology and Genetic Breeding, Jiangxi Agricultural University, Nanchang 330045, P. R. China
[3]School of Pharmacy, Jiangxi Science and Technology Normal University, Nanchang 330013, P. R. China
*E-mail: xujingkun@tsinghua.org.cn

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In view of the merits of the commercially available poly(3,4-ethylenedioxythiophene): poly(styrenesulfonate) (PEDOT:PSS), silver nanoparticles (AgNPs), and horseradish peroxidase (HRP), a stable sandwich-type biosensor was successfully constructed for the determination of hydrogen peroxide (H_2O_2). AgNPs was electrochemically deposited on the surface of water-stable PEDOT:PSS-Nafion films by cyclic voltammetry (CV). HRP was immobilized between AgNPs/PEDOT:PSS-Nafion composites and Nafion films. The response currents have a good linear relationship in the range from 0.05 to 20 μM, with a limit of detection of 0.02 μM (S/N = 3). Moreover, the biosensor exhibited a good stability and sensitivity. Satisfactory results showed that such sandwich-type Nafion/HRP/AgNPs/PEDOT:PSS-Nafion films could provide a promising platform for the biosensor designs and shows excellent electrocatalytic ability for H_2O_2.

Keywords: PEDOT:PSS; Electrochemical biosensor; hydrogen peroxide; Horseradish peroxidase; Silver nanoparticle;

1. INTRODUCTION

Hydrogen peroxide (H_2O_2), as one of the representative of reactive oxygen species (ROS) in biological systems, can be generated by photorespiration, mitochondrial electron transfer, or β-oxidation of fatty acid in plants. It is endogenous harmful substances in plant cells, which can damage

proteins, lipids and nucleic acids [1]. H_2O_2 plays an important role in many physiological processes [2, 3], such as dealing with adversity resistance and defense reaction, regulation of plant growth and development, and participate in the defense of the cell stomatal movement. Additionally, H_2O_2 has interaction with some plant hormones, including salicylic acid, auxin, and cytokinin, and also has an effect on a series of downstream signaling molecules (protein kinase and protein phosphatase). Therefore, a rapid and sensitive method for the determination of H_2O_2 is very necessary.

Conducting polymers (CPs) are fascinating materials that have been widely used in a variety of applications by virtue of their unique electrical, optical, and mechanical transduction mechanisms [4, 5]. Especially, due to easy processing, biocompatibility, and conductivity, CPs or the composites of CPs with nanomaterials (such as carbon nanotubes, metal particles or graphene) have been successfully employed in electrochemical biosensors [6-8]. In the development of biosensors, CPs or its composite material has been widely used as matrices for enzyme immobilization. As one of the most successful commercially available CPs, poly(3,4-ethylenedioxythiophene):poly(styrenesulfonate) (PEDOT:PSS) has been recently studied for biosensing application. One of outstanding properties of PEDOT:PSS is its ability to be easily doped and modified in order to obtain desired electrical and physical properties [9-11]. Shi et al. [12] have developed a counter electrode of dye-sensitized solar cells based on PEDOT:PSS-graphene nanocomposites deposited on indium tin oxide (ITO) substrates by spin coating. Lee et al. [13] have fabricated a new modified electrode by dispersing gold nanoparticles (AuNPs) onto the matrix of PEDOT:PSS for the determination of β-nicotinamide adenine dinucleotide. Xian et al. [14] indicated that PEDOT:PSS/Au nanocomposite was an excellent biocompatible platform for enzyme immobilization. Wen et al. [15] studied that Pt particles-loaded PEDOT:PSS matrix showed a high electrocatalytic activity and excellent stability for the oxidation of methanol. These findings suggest PEDOT:PSS may be a promising candidate for various sensing applications.

In recent years, metal nanoparticles have been the focus of much current research due to their large surface-to-volume ratio, strong adsorption ability, good electrical properties, high surface reaction activity, small particle size and good surface properties [16, 17]. Because of these unique properties, many metal nanoparticles have been wildly used in designing and in construction of enzyme biosensors. In particular, silver nanoparticles (AgNPs) have gained in popularity and have been widely used in the fabrication of electrochemical biosensors in recent decades because of unique properties such as biocompatibility, unique electronic and catalytic properties [18, 19]. Recently, several studies indicated that AgNPs exhibited catalytic activity for the reduction of H_2O_2 [19-21]. Meanwhile, horseradish peroxidase (HRP) has been proved to be the most commonly used enzyme for construction of hydrogen peroxide (H_2O_2) biosensors due to its widespread commercial availability at high purity and low cost [22]. Therefore, it is very meaningful to construct a H_2O_2 biosensor by incorporating the merits of AgNPs and HRP.

In our previous work, a lowly swelling and disintegrating PEDOT:PSS composite films as the electrochemical biosensing platform was successfully developed by incorporation of the commercially available PEDOT:PSS and biocompatible binding agent Nafion [23]. In order to obtained immobilize functionally active enzyme, a stable sandwich-type amperometric biosensor was successfully fabricated for detection of L-ascorbic acid (AA) based on poly(3,4-ethylenedioxythiophene) (PEDOT)-

single walled carbon nanotubes (SWCNT)/ascorbate oxidase (AO)/Nafion films [8]. Therefore, in this work, in view of the merits of the water-stable PEDOT:PSS-Nafion composites, AgNPs, and HRP, we constructed a stable sandwich-type biosensor for the determination of H_2O_2 by immobilizing the HRP between AgNPs/PEDOT:PSS-Nafion compistes and Nafion films. AgNPs were well-dispersed on PEDOT:PSS-Nafion matrix which can overcome the limitation of aggregation and nonspecific binding. Meanwhile, the conductivity, catalytic activity, and surface morphology of PEDOT:PSS-Nafion were all changed. In addition, AgNPs/PEDOT:PSS-Nafion compistes could provide an ideal microenviroment for biomacromolecule immobilization due to its aqueous compatibility and biocompatibility. The sandwich-type Nafion/HRP/AgNPs/PEDOT:PSS-Nafion biosensor was successfully applied for hydrogen peroxide biosensing.

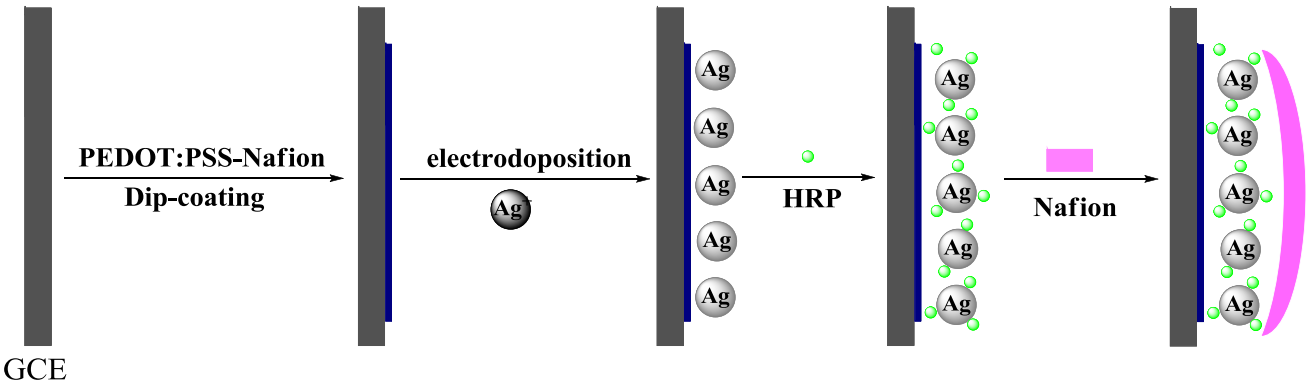
2. EXPERIMENTAL

PEDOT:PSS aqueous dispersion (Baytron P, 1.3 wt%) was purchased from Bayer AG. 5 wt% Nafion solution was obtained from DuPont Company. (HRP, RZ>3.0, A>250 U/mg) was purchased from Beijing Biodee Biotechnology Co., Ltd. Hydrogen peroxide (H_2O_2, 30%) was got from Tianjin Kermel Chemical Reagent Co., Ltd. Sliver nitrate (AgNO3) was purchased from Beijing Chemical Industry Co., Ltd. All other reagents were of analytical grade and obtained from the Sinopharm Chemical Reagent Company. Phosphate buffer solutions (PBS, 0.1 M) were used as a supporting electrolyte. All solutions were prepared using double-distilled deionized water.

Electrochemical experiments were performed on a CHI660B electrochemical workstation (Shanghai Chenhua Instrument Company, China) with a conventional three-electrode system. The bare glass carbon electrode (GCE) or composition modified electrode was served as the working electrode, a platinum wire with a diameter of 1 mm as the counter electrode, and the saturated calomel electrode (SCE) as the reference electrode. The addition of sample was carried out with a micropipettor (Dragonmed, Shanghai, China). Various pH values were measured with a Delta 320 pH meter (Mettler-Toledo Instrument, Shanghai, China). Ultraviolet-visible (UV-vis) spectra were measured with a Perkin-Elmer Lambda 900 UV-visible-near-infrared spectrophotometer. Electrochemical impedance spectra (EIS) results were recorded with Autolab Frequency Response Analyzer (AUT30, FRA2-Autolab, Eco Chemie, BV, Netherlands). Scanning electron microscopy (SEM) measurements were taken with a Hitachi S3000-N scanning electron microscope.

Before preparation of hydrogen peroxide sensor, the bare glassy carbon electrode (GCE) was continuously polished to a mirror-shine surface with 0.05 μm alumina slurry. Then the electrode was ultrasonically in doubly-distilled deionized water and ethanol in turn. PEDOT:PSS-Nafion composites were prepared by mixing 5 wt% Nafion solution with 1.3 wt% PEDOT:PSS aqueous dispersion in the volume rate of 1:1, and then stirred thoroughly for 24 h at room temperature in order to obtain homogenous suspension. 5 μL of this homogenous PEDOT:PSS-Nafion composites solution was directly dip-coated on GCE surface. After dried at room temperature under clean environment, the PEDOT:PSS-Nafion/GCE was electrochemically deposited by cyclic voltammetry (CV) at the scan rate of 100 mV s^{-1} from –1.0 V to +1.0 V *vs*. SCE in 0.1 M $AgNO_3$ solution. Then washed the

composite electrode repeatedly with double-distilled deionized water and naturally dried at room temperature. The sliver nanoparticles (AgNPs) composite electrode (AgNPs/PEDOT:PSS-Nafion/GCE) was obtained. 5 μL 3 mg/mL HRP was immobilized on the electrode surface to prepare composite electrode HRP/AgNPs/PEDOT:PSS-Nafion/GCE. After that, 5 μL 5 wt% Nafion was covered on the electrode surface to prevent the enzyme leakage [5]. Preparation process of Nafion/HRP/AgNPs/PEDOT:PSS-Nafion/GCE composite electrode is illustrated in Scheme 1. All prepared electrodes were stored in refrigerate at 4 ^{o}C when not in use.



Scheme 1. Preparation process of Nafion/HRP/AgNPs/PEDOT:PSS-Nafion/GCE composite electrode.

3. RESULTS AND DISCUSSION

3.1. Morphology characterization of PEDOT:PSS composite films

SEM was employed to investigate the surface morphology of (a) PEDOT:PSS-Nafion, (b) HRP/PEDOT:PSS-Nafion, (c) AgNPs/PEDOT:PSS-Nafion, and (d) HRP/AgNPs/PEDOT:PSS-Nafion films. As seen from Fig. 1A, PEDOT:PSS-Nafion films presented a rough surface consisting of some little pore, which could provide a good environment to disperse and fix the individual catalyst nanoparticales [24]. These little pores distributed relatively homogeneous on the surface of PEDOT:PSS-Nafion films, indicating PEDOT:PSS aqueous dispersion and Nafion solution has been mixed well and a homogeneous black suspension was formed. SEM image of HRP/PEDOT:PSS-Nafion films was showed in Fig. 1B. HRP uniformly distributed on PEDOT:PSS-Nafion films, and indicated that the good biocompatibility of PEDOT:PSS-Nafion can be served as a matrix for biomolecules. Fig. 1C shows SEM image of the as-prepared AgNPs/PEDOT:PSS-Nafion films after the electrodeposition process. AgNPs were homogeneous distributed and fixed tightly on PEDOT:PSS-Nafion films, indicating that AgNPs have been successfully electrodeposited on PEDOT:PSS-Nafion films. At high magnification, it can be seen that AgNPs had a round shape and were highly crystalline, with diameters around 50-200 nm, which was benefit for providing more active sites [25]. From Fig. 1D, at low magnification, HRP and AgNPs combined into piece and homogeneous distributed on PEDOT:PSS-Nafion films. High magnification revealed that three or

more hydrogen peroxide enzyme molecules wrapped around every AgNPs. The diameter of HRP-AgNPs was around 500 nm, which was important to increase active surface area.

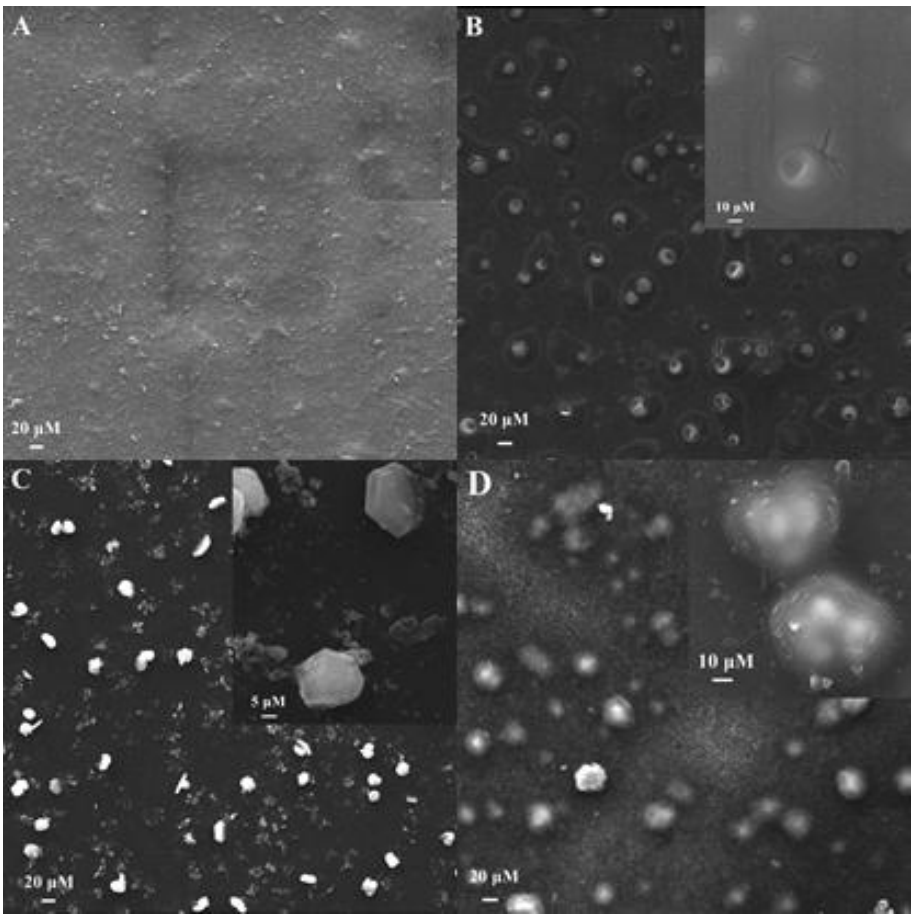


Figure 1. SEM images of (a) PEDOT:PSS-Nafion, (b) HRP/PEDOT:PSS-Nafion, (c) AgNPs/PEDOT:PSS-Nafion, (d) Nafion/AgNPs/HRP/PEDOT:PSS-Nafion composite films.

3.2. UV-vis absorption spectra study

UV-vis spectra of Nafion (a), PEDOT:PSS (b), PEDOT:PSS-Nafion (c), HRP (d), and HRP/PEDOT:PSS-Nafion (e) solution are illustrated in Fig. 2. UV-vis spectra of AgNPs/PEDOT:PSS-Nafion (f) and HRP/AgNPs/PEDOT:PSS-Nafion (g) polymer films on ITO electrodes were illustrated in Fig. 2 (inset). As shown in Fig. 2, no absorption was observed in Nafion solution (curve a). Whereas, one broad band at 450-900 nm range was observed from PEDOT:PSS aqueous dispersion (curve b), which was attributed to the polarons and bipolarons of the PEDOT [26]. After mixing PEDOT:PSS aqueous dispersion with Nafion solution, the characteristic absorption of PEDOT:PSS still could be observed in the same region (curve c). The same result was also obtained when electrodeposited AgNPs on the surface of PEDOT:PSS-Nafion films (curve f). Absorption peak of HRP in 0.1 M PBS (pH 7.0) was appeared at 403 nm (curve d). After mixing HRP with PEDOT:PSS-Nafion solution, the absorption peak was also appeared at 403 nm (curve e). Similarly,

the absorption peak of HRP/AgNPs/PEDOT:PSS-Nafion films was appeared at 401 nm (curve g), with only 2 nm shifts, indicating that HRP in PEDOT:PSS-Nafion composite or on HRP/AgNPs/PEDOT:PSS-Nafion films was not distinctly denatured and its secondary structure was kept as the native state of HRP [27].

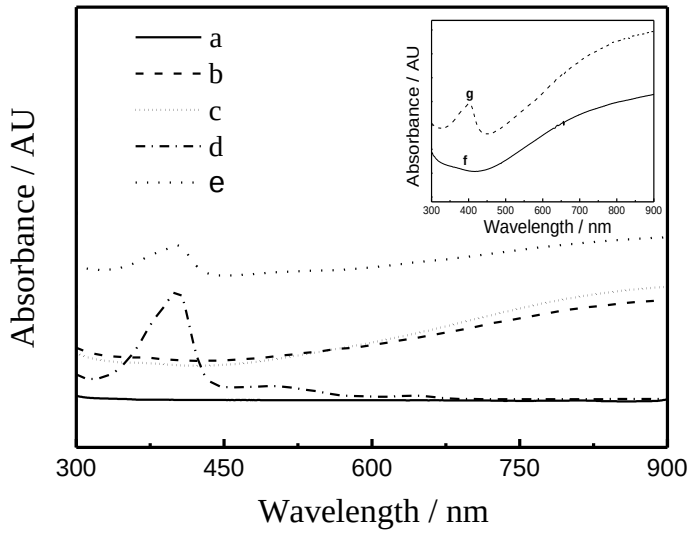


Figure 2. UV-vis absorption spectra of (a) Nafion, (b) PEDOT:PSS, (c) PEDOT:PSS-Nafion mixture, (d) HRP in pH 7.0 PBS, and (e) HRP in PEDOT:PSS-Nafion mixture. Inset: UV-vis absorption spectra of (f) AgNPs/PEDOT:PSS-Nafion and (g) Nafion/HRP/AgNPs/PEDOT:PSS-Nafion.

3.3. Electrochemical impedance spectra (EIS) analysis

EIS were performed to provide information on the interfacial electron-transfer kinetics of different modified electrodes. Fig. 3 shows the EIS of bare GCE, PEDOT:PSS-Nafion/GCE, Nafion/HRP/PEDOT:PSS-Nafion/GCE, AgNPs/PEDOT: PSS-Nafion/GCE, and Nafion/HRP/AgNPs/PEDOT:PSS-Nafion/GCE. The impedance of bare GCE was 905 Ω (curve a). After PEDOT:PSS-Nafion coated on the surface of GCE, the EIS of PEDOT:PSS-Nafion/GCE was decreased to 37 Ω (curve b), which was attributed to the high conductivity of PEDOT:PSS that facilitated the electron transfer on the electrode surface. However, HRP immobilized on the PEDOT:PSS-Nafion/GCE surface, a remarkable increase in the electron-transfer resistant (512 Ω, curve c) was resulted, due to the presence of HRP blocked the electron-transfer. When AgNPs were electrodeposited on the PEDOT:PSS-Nafion/GCE surface, a very low electron-transfer resistance (26 Ω, curve d) was obtained. This result could be attributed to the increase conductivity of AgNPs. After the AgNPs/PEDOT:PSS-Nafion/GCE was coated with HRP, the electron-transfer resistance was increased to 60 Ω (curve d), revealing the coverage of the enzyme on the composite surface.

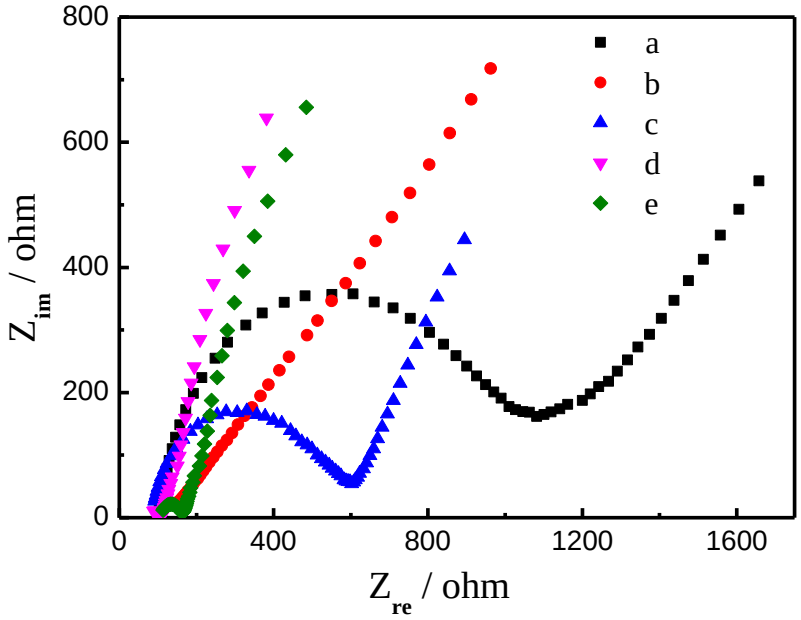


Figure 3. EIS for (a) bare GCE, (b) PEDOT:PSS-Nafion/GCE, (c) Nafion/HRP/PEDOT:PSS-Nafion/GCE, (d) AgNPs/PEDOT:PSS-Nafion/GCE, (e) Nafion/AgNPs/HRP/PEDOT:PSS-Nafion/GCE, in 5 mM $[Fe(CN)_6]^{4-/3-}$ containing 0.1 M KCl. The frequencies swept from 100 mHz to 10 kHz

3.4. Electrochemical behavior of H_2O_2

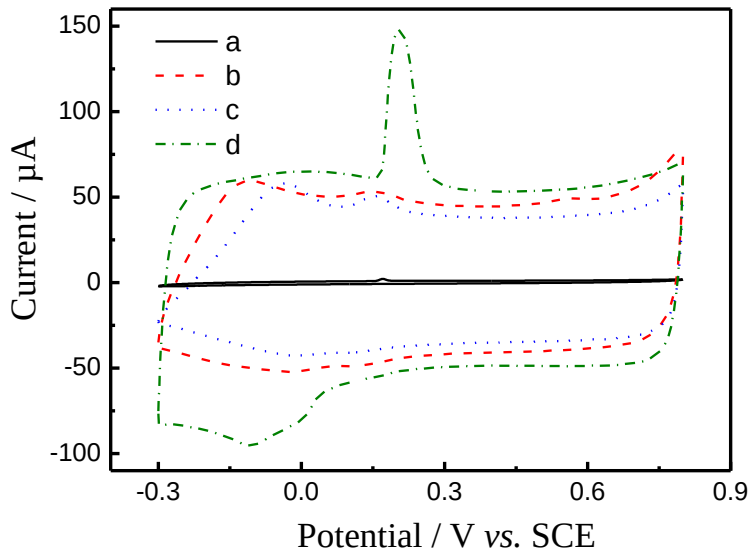


Figure 4. CVs of 40 μM H_2O_2 in 0.1 M PBS (pH 7.0) at different electrodes: (a) bare GCE, (b) PEDOT:PSS-Nafion/GCE, (c) Nafion/HRP/PEDOT:PSS-Nafion/GCE, (d) Nafion/AgNPs/HRP/PEDOT:PSS-Nafion/GCE. Scan rate: 50 mV s^{-1}.

Fig. 4 shows the cyclic voltammograms (CVs) of H_2O_2 at different composite modified electrodes with scan rate of 50 mV s^{-1} in 0.1 M PBS (pH 7.0). CVs of PEDOT:PSS-Nafion/GCE showed a poor and broad oxidation peak (curve b) compared with bare GCE (curve a). For Nafion/HRP/PEDOT:PSS-Nafion/GCE, a oxidation peak was exhibited at 0.155 V (curve c). This could be attributed to the catalytic of HRP. However, the electrochemical response at Nafion/HRP/AgNPs/PEDOT:PSS-Nafion/GCE was dramatically enhanced, with the oxidation peak at 0.201 V (curve d). On the one hand, this was because AgNPs were homogenously distributed on the PEDOT:PSS-Nafion surface and provided a three-dimensional interlaced porous network for immobilization of enzyme (HRP), on the other hand, owing to the unique electrocatalytic properties, large surface area and good conductivity of AgNPs [18, 19, 28]. The synergetic effect of HRP and AgNPs exhibited an excellent electrocatalytic activity for H_2O_2.

3.5. Effect of pH value and scan rate

The effect of pH value on the response of Nafion/HRP/AgNPs/PEDOT:PSS-Nafion/GCE was investigated between 3.0 to 9.0 in the present 40 μM H_2O_2. As shown in Fig. 5, the currents response increased gradually with the increasing pH value, and attained a maximum at pH 7.0. Then, the currents response decreased rapidly when pH increased further. This result was in agreement with soluble HRP [29], implying that the AgNPs/PEDOT:PSS-Nafion matrix did not alter the optimal pH value for electron transfer of immobilized HRP to H_2O_2. Therefore, in order to obtain high sensitivity and selectivity, pH 7.0 was selected as the optimum pH for the detection of H_2O_2.

CVs of Nafion/HRP/AgNPs/PEDOT:PSS-Nafion/GCE in 0.1 M PBS (pH 7.0) at different scan rates are shown in Fig. 6A. With the increase of scan rate from 20 to 200 mV s^{-1}, the oxidation peak current (I_{pa}) was proportional to the scan rate (ν) (Fig. 6B). The regression equation was I_{pa} (μA) = 61.37 + 1.73 ν (ν in mV s^{-1}), with a correlation coefficient of 0.998, suggesting that the electron-transfer process for Nafion/HRP/AgNPs/PEDOT:PSS-Nafion/GCE was a typically surface-controlled process. The anode peak potential (E_{pa}) had a linear relationship with the logarithm of scan rate (ln ν) (Fig. 6C), the regression equation was E_{pa} (V) = 0.0696 – 0.0345 ln ν (ν in mV s^{-1}) (R^2 = 0.9975). According to the Laviron's equation [30]:

$$E_{pa} = E^0 + [RT / (1-\alpha) nF] \ln [RTk_s / (1-\alpha) nF] – [RT/ (1-\alpha) nF] \ln \nu \quad (1)$$

Taking a charge transfer coefficient α of 0.5, the number of electrons transferred n was calculated to be 2. The electron transfer rate constant (*ks*) can be obtained based on the equation [31]:

$$\log k_s = \alpha \log(1 – \alpha) + (1 – \alpha) \log\alpha – \log RT/nF\nu – (1 – \alpha)\alpha F\Delta E_p /(2.03RT) \quad (2)$$

Here, α is the charge transfer coefficient, R, T and F have their conventional meanings. ΔE_p is the peak to peak potential separation. Taking a charge transfer coefficient α of 0.5 and a scan rate of 20 mV s^{-1}, and k_s was calculated to be 1.14 s^{-1}.

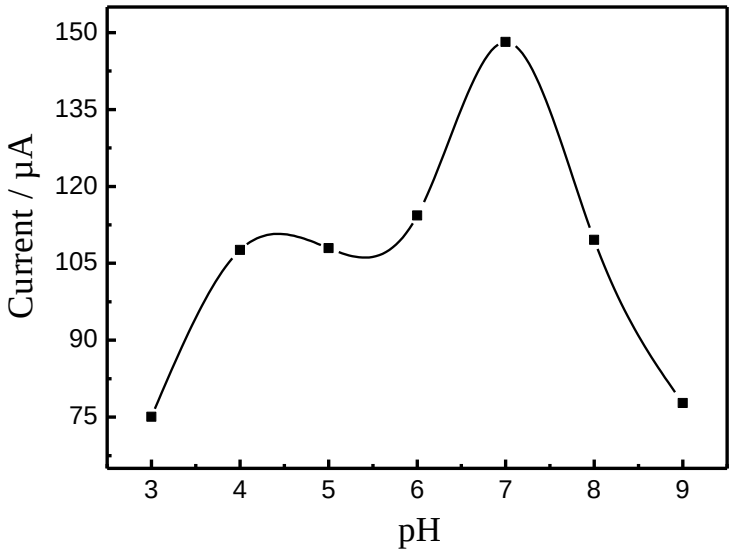


Figure 5. Effect of pH on the oxidation peak currents of Nafion/AgNPs/HRP/PEDOT:PSS-Nafion/GCE in 0.1 M PBS (pH 7.0) containing 40 μM H_2O_2. Scan rate: 50 mV s^{-1}.

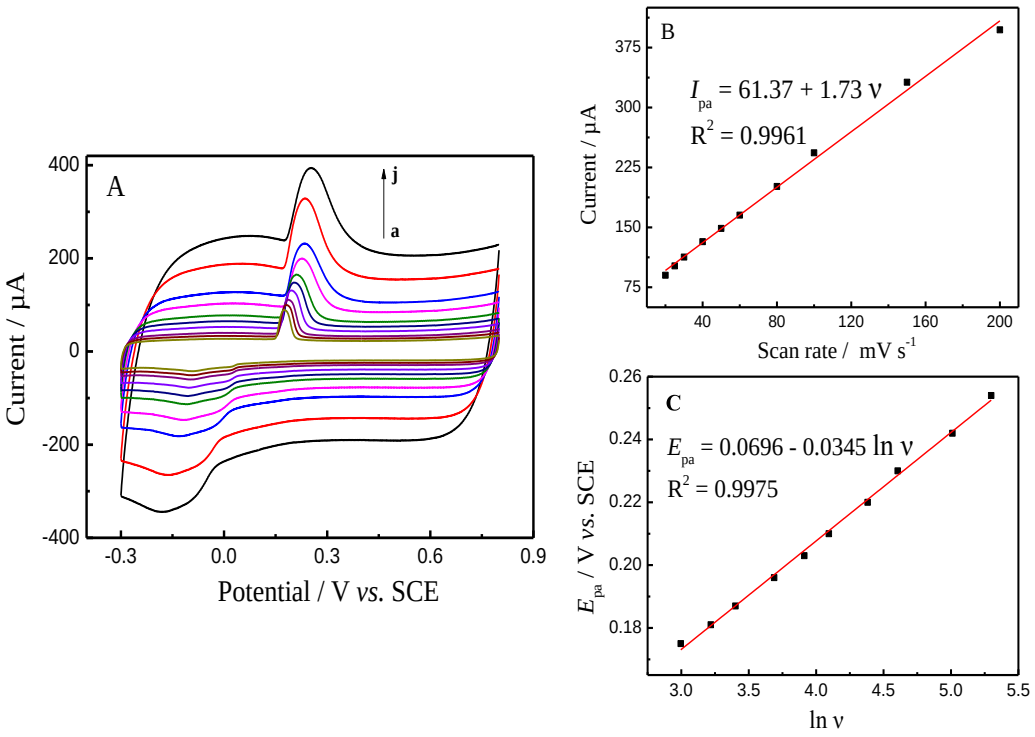


Figure 6. (A) CVs of 40 μM H_2O_2 in 0.1 M PBS (pH 7.0) with different scan rates: 20, 25, 30, 40, 50, 60, 80, 100, 150, and 200 mV s^{-1} (from a to j). (B) The plot of anodic peak currents *vs.* scan rate.

3.6. Electrocatalytic reduction of H_2O_2

The electrocatalytic activity of Nafion/HRP/AgNPs/PEDOT:PSS-Nafion/GCE toward H_2O_2 was investigated by DPV. As shown in Fig. 7, the peak currents were linearly proportional to H_2O_2

concentrations in the ranges of 0.05-1.0 μM and 1.0-20 μM, its linearization equations were I_1 (μA) = 21.34 C (μM) + 98.28 (R = 0.9938) and I_2 (μA) = 2.30 C (μM) + 116.58 (R = 0.9994), respectively.

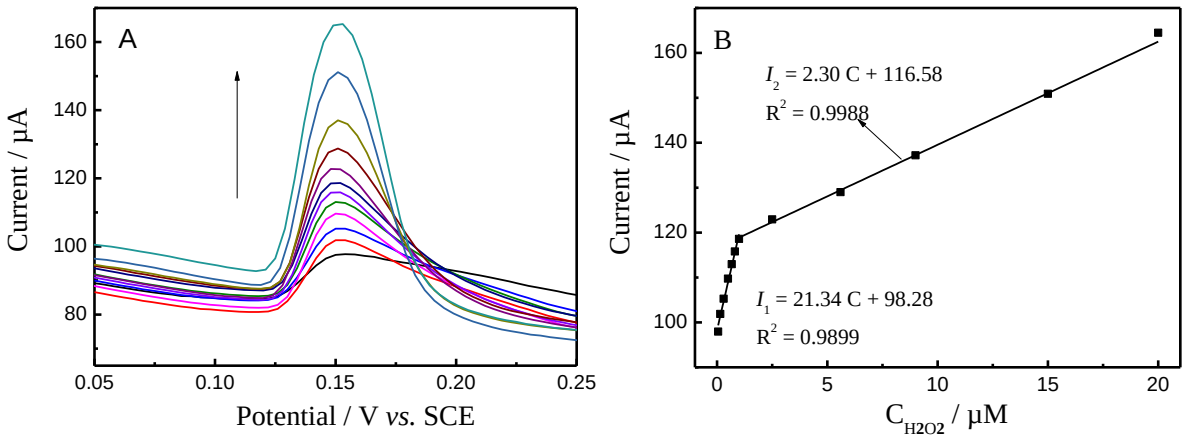


Figure 7. (A) DPVs of various concentrations of H_2O_2 at Nafion/HRP/AgNPs/PEDOT:PSS-Nafion/GCE in 0.1 M PBS (pH 7.0). H_2O_2 concentrations (from bottom to top): 0.05, 0.15, 0.3, 0.5, 0.67, 0.81, 1.0, 2.5, 5.6, 9, 15, and 20 μM. Scan rate: 50 mV s^{-1}. (B) The linear relationship between peak current and concentration of H_2O_2.

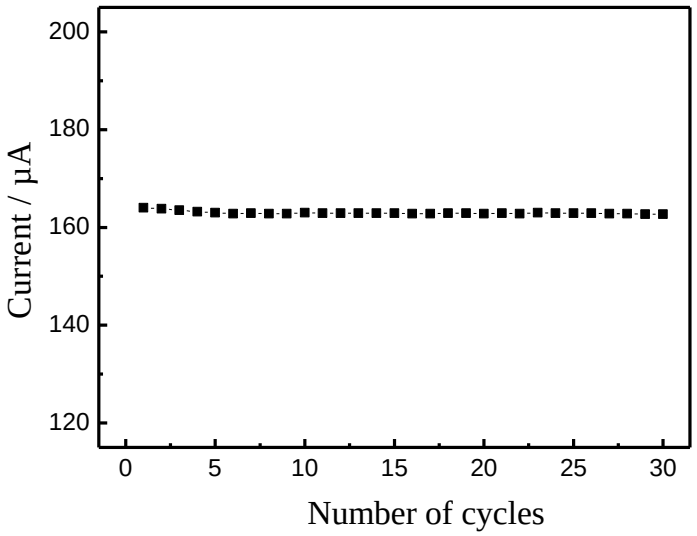


Figure 8. The stability of Nafion/HRP/AgNPs/PEDOT:PSS-Nafion/GCE containing 40 μM H_2O_2 in 0.1 M PBS (pH 7.0). Scan rate: 50 mV s^{-1}.

The detection limit was 0.02 μM (S/N = 3), which can be comparable to electrochemical reduction of grapheme oxide-aminothiophenol (ERGO-ATP)-Pd/GCE [32] and HRP/PTMSPA @ GNR [33] or even better than AgNPs/DNA/GCE [19], Nafion-HRP/PS-MWCNT/Au [34], HRP/PEDOT-PSS-Au/GCE [22], AgNPs/SnO_2/GCE [35], AgNPs-SWCNT/PET [36], and ITO/APTMS/HRP [25] (Table 1). This result indicated Nafion/HRP/AgNPs/PEDOT:PSS-Nafion/GCE had better electrocatalytic activity.

The reproducibility of Nafion/HRP/AgNPs/PEDOT:PSS-Nafion/GCE was estimated. There was no obvious change of the redox currents by 30th successive measurements. The relative standard deviation (RSD) was 0.45%. When the electrode was kept at 4 °C for two weeks, the respons of biosensor still retains 93% of their initial values. The above results revealed that Nafion/HRP/AgNPs/PEDOT: PSS-Nafion/GCE had a good stability.

Table 1. Comparison of the different modified electrodes for determination of H_2O_2

Electrode	Electrolyte	Linear range	Limit of detection (μM)	Reference
ERGO-ATP-Pd/GCE	0.1 M PBS (pH 7.4)	0.1 μM-10 mM	0.016	[32]
HRP/PTMSPA@GNR	0.1 M PBS (pH 7.0)	10 μM-1.0 mM	0.06	[33]
AgNPs/DNA/GCE	0.2 M PBS (pH 7.0)	4.0 μM-16.0 mM	1.7	[19]
Nafion-HRP/PS-MWCNT/Au	0.1 M PBS (pH 7.0)	0.5 μM-0.82 mM	0.16	[34]
HRP/PEDOT-PSS-Au/GCE	0.2 M PBS (pH 7.2)	0.2 μM-0.38 mM	0.10	[22]
AgNPs/SnO_2/GCE	0.1 M PBS (pH 7.0)	0.01μM-35.0 μM	5.0	[35]
AgNPs-SWCNT/PET	0.1 M PBS (pH 7.4)	0.016-18.08 mM	2.76	[36]
ITO/APTMS/HRP	0.1 M PBS (pH 7.0)	0.02-8 mM	8.0	[25]
Nafion/HRP/AgNPs/ PEDOT:PSS-Nafion/GCE	0.1 M PBS (pH 7.0)	0.05-20 μM	0.02	This work

APTMS: (3-aminopropyl)trimethoxysilane; PS: polystyrene; GNR: gold nanorods; PTMSPA: poly(N-[3-(trimethoxysilyl)propyl]aniline; ATP: aminothiophenol; ERGO: electrochemical reduction of grapheme oxide;

4. CONCLUSIONS

A stable sandwich-type biosensor was successfully constructed for the determination of H_2O_2 with the merits of commercially available PEDOT:PSS, AgNPs, and HRP. The electrochemical responses of Nafion/HRP/AgNPs/PEDOT:PSS-Nafion modified electrode toward to H_2O_2 shows superior performance such as good sensitivity, wide linear range, low detection limit, and good stability, indicating that AgNPs/PEDOT:PSS-Nafion matrix could provide a promising platform for enzyme immobilization and biosensor designs. Such a sandwich-type biosensor could also provide a promising platform for practical applications in agro-industry

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