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A Novel Hydrogen Peroxide Biosensor Based on Horseradish Peroxidase Immobilized on Poly(aniline-*co*-*o*-aminobenzoic acid) Modified Glassy Carbon Electrode Coated with Chitosan Film

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In this work, a novel amperometric biosensor for hydrogen peroxide (H_2O_2) determination was successfully prepared by immobilizing horseradish peroxidase (HRP) on poly(aniline-*co*-*o*-aminobenzoic acid) or p(Ani-*co*-*o*-Aba) and then covered with chitosan (CS) film. The immobilized HRP displayed an excellent electrocatalytic activity to the reduction of hydrogen peroxide. The effects of experimental variables such as the *o*-Aba mol ratios in p(Ani-*co*-*o*-Aba) synthesis, HRP and CS concentrations, pHs of supporting electrolyte solution and applied potentials for the working electrode were investigated for the optimized conditions. This novel biosensor exhibits a fast response toward H_2O_2 with a linear range from 10 to 1,000 μM and a detection limit of 1.8 μM based on the signal-to-noise ratio (S/N = 3). The developed biosensor shows satisfactory tolerance with other potential interferences such as dopamine (DA), ascorbic acid (AA), glucose (Glu) and uric acid (UA), and also shows a good stability for ~2 weeks.

Keywords: Hydrogen peroxide biosensor, Horseradish peroxidase, Poly(aniline-*co*-*o*-aminobenzoic acid), Glassy carbon electrode, Chitosan

1. INTRODUCTION

Hydrogen peroxide (H_2O_2) is extremely reactive species which is an enzymatic intermediate substance obtained from many enzyme-substrate reactions [1]. The determination of H_2O_2 is very important in food, industrial, clinical and environmental assays [2-3]. Many scientific methodologies have been widely employed for determination of H_2O_2, such as titrimetry, spectrophotometry,

fluorimetry, chemiluminescene and electroanalytical method [4-7]. Among these techniques, the electroanalytical method is advantageous over other methods because of their robustness, possible miniaturization, low-cost and ease of operation. Due to their high selectivity and sensitivity, the development of biosensors based on peroxidase-modified electrodes has been widely studied in the field of electroanalytical chemistry [8].

The horseradish peroxidase (HRP), a heme-containing oxidoreductase, is a commercially important enzyme, which catalyzes the reductive cleavage of H_2O_2 by an electron donor [9]. However, the sensitivity of these biosensors based on the direct electron exchange between HRP and electrode surface is often relatively low. To increase the current response, HRP is widely fixed onto the electrode surface with the metal nanoparticles, carbon nanomaterials, and conducting polymers [10-12].

Polyaniline and its derivatives have been of the most attractive conducting polymers due to their easy synthesis, low cost and high environmental stability, and variable electrical conductivity, excellent redox recyclability and optical activity [13-16]. They easily facilitates not only the electron transfer between the enzyme and electrode surface, but also make a more convenient environment for localizing the enzyme onto micro-size electrode surfaces in the electrochemical biosensors.

Chitosan (CS) is obtained from the partial deacetylation of chitin as one of the most abundant polysaccharides in the world [17]. CS is a biocompatible biopolymer possessing diverse properties for numerous applications, such as film-forming ability, hydrophilicity, high mechanical strength, good adhesion and chemical inertness [18]. Because of this unique combination of properties, there have been several reports using CS as ionically immobilizing and physically coating materials for preparing enzyme biosensors [19-21].

The design of a novel enzyme biosensor based on conducting-functionalized polymers and polymer-coated supports remain a significant focus of the study to consider in the enzyme immobilization strategy. In this study, the poly(aniline-*co*-*o*-aminobenzoic acid) or p(Ani-*co*-*o*-Aba) having different carboxylic (–COOH) groups were prepared by copolymerization of aniline (Ani) and *o*-aminobenzoic acid (*o*-Aba). Then, the HRP immobilized on p(Ani-*co*-*o*-Aba) was casted onto the glassy carbon electrode (GCE) by a simple drop-coating method. The outer layer CS film as a binder was further coated on the electrode surface. We found that the CS/HRP-p(Ani-*co*-*o*-Aba)/GCE shows an excellent activity for the electrocatalysis of H_2O_2 reduction. To explore its analytical performances, the proposed biosensor was used for the determination of H_2O_2 over other interfering substances.

2. MATERIALS AND METHODS

2.1 Reagents

Horseradish peroxidase (HRP, EC 1.11.1.7, M_r ~ 40,000 (Lit.), 969.65 U mg^{-1}), ammonium persulphate ($(NH_4)_2S_2O_8$) and hydrogen peroxide (H_2O_2, 30%) were purchased from Fluka. Aniline (Ani), *o*-aminobenzoic acid (*o*-Aba) and chitosan (CS) were purchased from Sigma-Aldrich. All other chemicals used in this study were of analytical grade and used as received, except for the aniline which

was purified by double distillation under reduced pressure prior to use and stored at 4 °C in the fridge when not in use. All aqueous solutions were freshly prepared using de-ionized (DI) water (R ≥ 18.2 MΩ cm) purified with a Nano-pore^{TM} ultrapure water system. A stock standard solution was prepared freshly each day. The concentration of H_2O_2 solution was determined by titration with $KMnO_4$ [4]. All measurements were carried out in 0.1 M phosphate buffer solution (PBS) used as supporting-electrolyte solution. The PBS with various pH values was prepared by mixing stock standard solutions of NaH_2PO_4 and Na_2HPO_4 and then adjusted the pH with NaOH or H_3PO_4.

2.2 Instruments and apparatus

All electrochemical measurements were carried out with an eDAQ potentiostat (EA161) equipped wit and e-corder (201) controlled by E-Chem software. A conventional three-electrode system was used with the modified electrode as working electrode, the Ag/AgCl (sat. 3.0 M KCl) as reference electrode, and a platinum (Pt) wire as counter electrode. A 713 pH meter (Metrohm, Switzerland) was used to monitor the pH of PBS. UV-Visible absorption spectra of solutions were recorded by using a Perkin Elmer Lambda25 UV-Visible spectrophotometer with the path length of a standard rectangular quartz cuvette (10 x 10 mm, Hellma Analytics) over the wavelength ranging from 300 to 800 nm. Atomic force microscope (AFM) images were obtained with scanning microscope probe (Park Systems Corp., Korea.) controlled by the XEI software.

2.3 Preparation of p(Ani-co-o-Aba)

A typical method of the copolymers synthesis described by Huang et al. [22-23] was adopted. Briefly, two monomers including Ani and *o*-Aba were dissolved in 5 mL of chloroform ($CHCl_3$). $(NH_4)_2S_2O_8$ used as the oxidant was dissolved in 5 mL of 1.0 M hydrochloric acid solution. After the reaction was left at room temperature for 24 h, the solid copolymer was collected from the reaction by filtration. To remove excess acid and unreacted monomers, the filtered solid product was then washed with several portions of diluted HCl, DI water and ethanol, respectively, and finally dried under vacuum. The structure of conducting p(Ani-*co*-*o*-Aba) from interfacial copolymerization is summarized in Figure1. When a carboxyl group is deprotonated, its conjugate base forms a carboxylate anion ($-COO^-$).

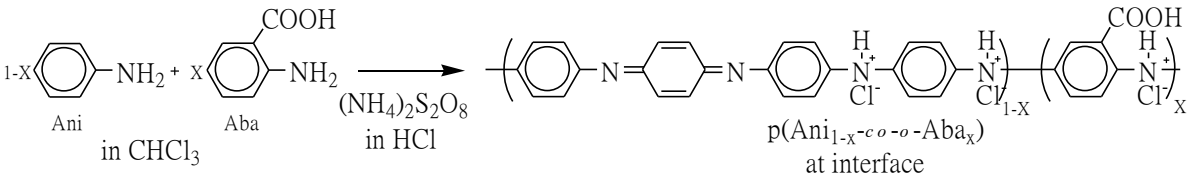

Figure 1. The structure of conducting p(Ani-*co*-*o*-Aba) prepared from Ani and *o*-Aba , where x ($0 \leq x \leq 1$) relates to the substitution stoichiometry of *o*-Aba unit in p(Ani-*co*-*o*-Aba).

2.4 Preparation of modified electrodes

The GCE (CH Instrument Co., USA) with a diameter of 3 mm was polished with 0.3-, 0.1-, and 0.05-μm alumina (Al_2O_3) slurry using a soft micro cloth polishing pad, and then thoroughly rinsed with DI water. After that, the GCE was ultrasonically treated sequentially in DI water for 10 min to remove residual polishing materials physically adsorbed on the electrode surface and then dried in air. The GCE was cleaned by potential cycling between –1.0 V and +1.0 V (*vs.* Ag/AgCl) at 50 mV s^{-1} in 0.1 M H_2SO_4 solution until the stable cyclic voltammograms (CVs) were obtained.

The preparation of H_2O_2 biosensor based on the CS/HRP-p(Ani-*co*-*o*-Aba)/GCE can be schematically illustrated in Figure 2. For the preparation of CS/HRP-p(Ani-*co*-*o*-Aba)/GCE, the HRP enzyme was mixed to the mixture before casting. Briefly, a 20 μL of 10 mg mL^{-1} homogeneous p(Ani-*co*-*o*-Aba) solution was mixed with an equivalent volume of 4 mg mL^{-1} HRP solution (Figure 2A). The positively charged HRP was immobilized on negatively charged p(Ani-*co*-*o*-Aba) by electrostatic attraction. After vortexing for 15 min, a 10 μL of the resulting homogeneous solution was pipetted onto the well-polished GCE surface, which was kept at room temperature for 3 h. According to the previous reports by Chico et al. [24] and Chen et al. [25], stable biocatalysts could be efficiently immobilized electrostatically in polysaccharide-coated supports. Then in our work, 5 μL of CS solution was dropped onto the surface of the HRP-p(Ani-*co*-*o*-Aba)/GCE to fabricate the H_2O_2 biosensor (Figure 2B). Finally, the CS/HRP-p(Ani-*co*-*o*-Aba)/GCE was air-dried for overnight at room temperature and kept at 4 °C in the fridge when not in use.

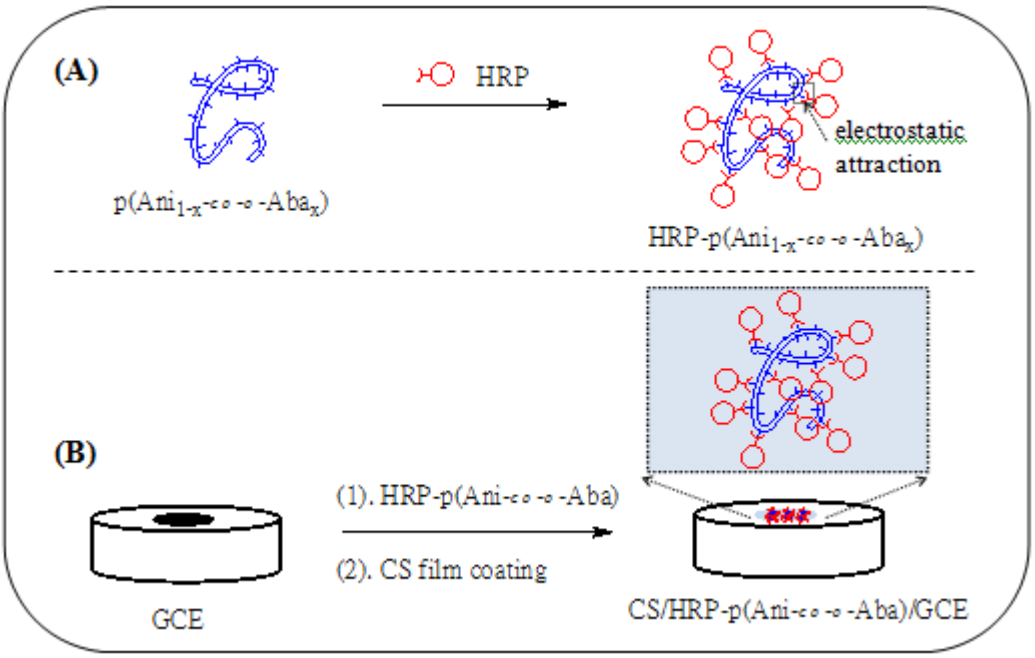

Figure 2. Preparation of H_2O_2 biosensor based on the CS/HRP-p(Ani-*co*-*o*-Aba)/GCE: (A) Immobilization of HRP using p(Ani-*co*-*o*-Aba). (B) Drop-coating method of HRP-p(Ani-*co*-*o*-Aba) onto GCE coated with an outer layer CS film.

2.5 Electrochemical measurements

All electrochemical measurements were carried out with eDAQ potentiostat (e-corder 401) controlled by EChem software. A conventional three-electrode system consisting of CS/HRP-p(Ani-*co*-*o*-Aba)/GCE working electrode, the Ag/AgCl (sat. 3.0 M KCl) reference electrode, and a platinum (Pt) wire counter electrode, was used throughout. The cyclic voltammetric and amperometric experiments were performed in a thermostatic electrochemical cell at 25 °C. The supporting electrolyte solutions were purged vigorously for at least 10 min to remove O_2 and kept under N_2 atmosphere during the measurements. The CS/HRP-p(Ani-*co*-*o*-Aba)/GCE was electrochemically performed by cycling the potential between –0.6 V and +0.4 V (*vs.* Ag/AgCl) in the absence or presence of H_2O_2. Amperometric experiments were carried out in a stirred batch system of 10 mL glass cell by applying a potential of -0.3 V to the working electrode and adding successively freshly prepared aliquot of H_2O_2 standard solution to the electrolyte solution. Current–time data were recorded after the steady state current to be achieved. The CS/HRP-p(Ani-*co*-*o*-Aba)/GCE was kept at 4 °C in a dry condition in the fridge when not in use.

3. RESULTS AND DISCUSSIONS

3.1 UV-Vis absorption spectra of p(Ani-co-o-Aba)

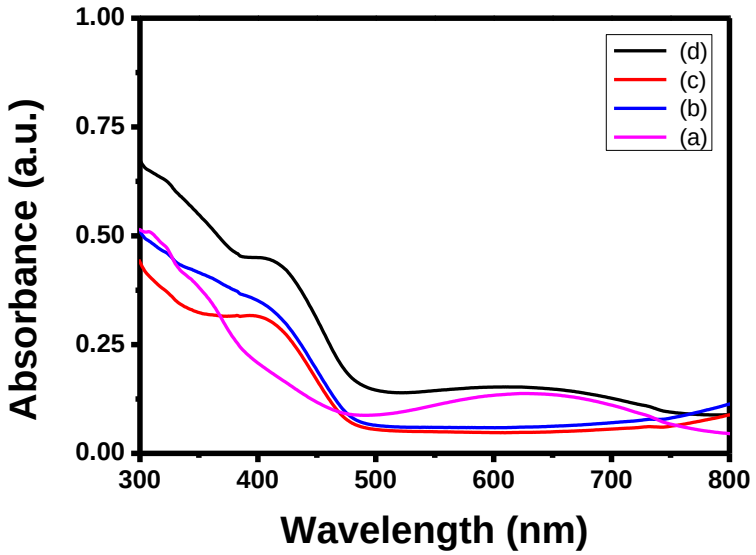

Figure 3. UV-Vis absorption spectra of p(Ani-*co*-*o*-Aba) for (a) 0.2, (b) 0.4, (c) 0.6 and (d) 0.8 mol ratio of *o*-Aba to Ani.

Considering the nature of carboxylic acid groups, the as-prepared p(Ani-*co*-*o*-Aba) could be soluble in an aqueous alkaline solution or in polar organic solvents such as DMF, DMSO, etc. In this study, a p(Ani-*co*-*o*-Aba) solution was prepared by dissolving each copolymer in DMSO. The

solubility increases with the increasing of *o*-Aba content in the copolymers. As shown in Figure 3, the UV-Vis absorption spectra showed two absorption bands over the wavelength of 400 - 700 nm, which is similar to the characteristics of polyaniline (see, [14,23,26]). With increasing the ratio of *o*-Aba in the copolymer composition, the spectra of the copolymers showed the strong absorption peak around 390 nm with varying intensities. The peak around 390 nm is ascribed to the π-π* transition of the benzenoid rings as reported for the absorption spectra of amino benzoic acid, while the other band around 620 nm is attributed to the n-π* transition from the nonbonding nitrogen lone pair of a localized benzenoid highest occupied molecular orbital (HOMO) to the π* of a quinoid lowest unoccupied molecular orbital (LUMO) [27].

3.2 AFM of p(Ani-co-o-Aba)/GCE, HRP-p(Ani-co-o-Aba)/GCE and CS/HRP-p(Ani-co-o-Aba)/GCE

The AFM technique was widely employed to investigate the surface topography of modified electrodes.

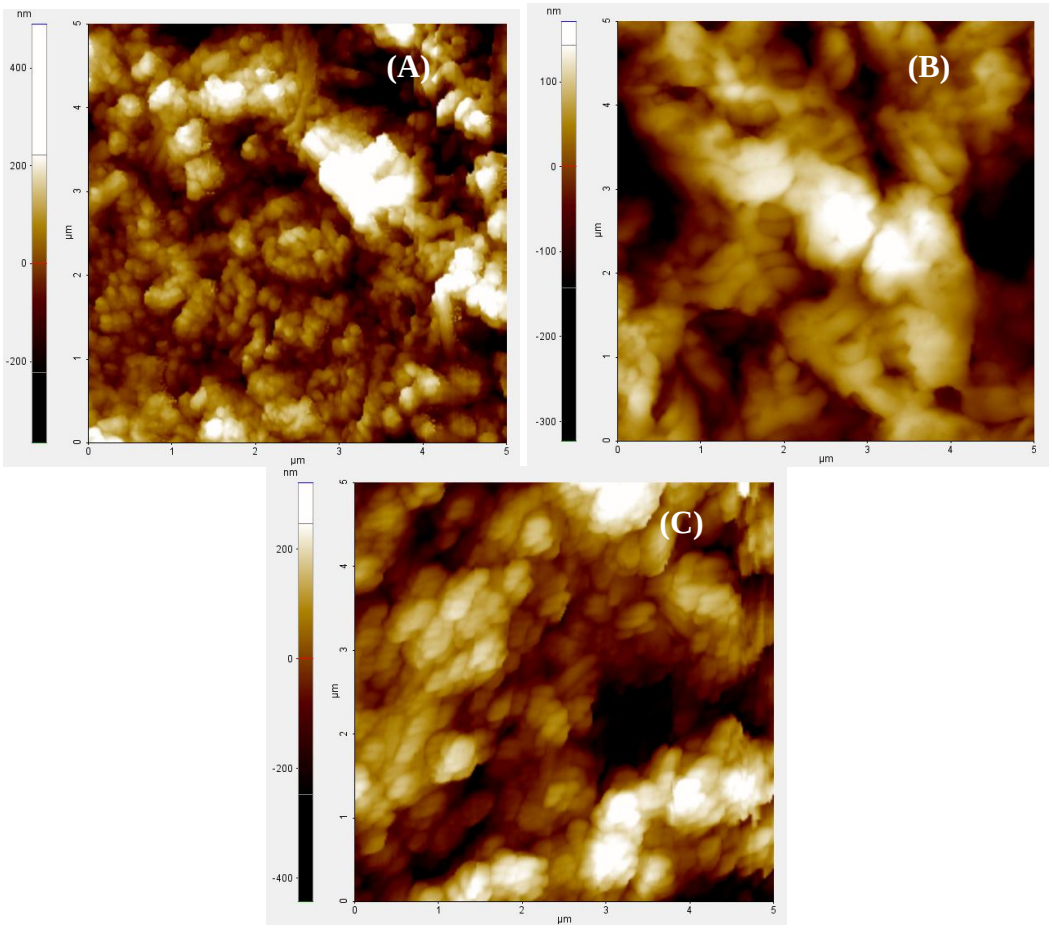

Figure 4. Representative AFM images of (A) p(Ani-*co*-*o*-Aba)/GCE, (B) HRP-p(Ani-*co*-*o*-Aba)/GCE and (C) CS/HRP-p(Ani-*co*-*o*-Aba)/GCE.

Figure 4A-C show the representative AFM image (5 μm x 5 μm scan size) of the p(Ani-*co*-*o*-Aba)/GCE, HRP-p(Ani-*co*-*o*-Aba)/GCE and CS/HRP-p(Ani-*co*-*o*-Aba)/GCE, respectively. As shown in Figure 4A, the p(Ani-*co*-*o*-Aba) particles deposited on GCE were in a range of a few hundred nanometers, which give rise to higher surface and easier immobilization of HRP molecules than the bare electrodes [23]. As shown in Figure 4B and 4C, the topographical images of the HRP-p(Ani-*co*-*o*-Aba)/GCE and CS/HRP-p(Ani-*co*-*o*-Aba)/GCE quite different from the p(Ani-*co*-*o*-Aba)/GCE were observed. This is attributed to the fact that immobilized HRP and CS film coating mainly changed the surface characteristic of the p(Ani-*co*-*o*-Aba)/GCE. These results obtained here were similar to several enzyme electrodes [28-29].

3.3 Electrochemical behavior of CS/HRP-p(Ani-co-o-Aba)/GCE

All electrochemical measurements were performed in a PBS (pH 6.5) to investigate the changes of the electrochemical behavior after each modification step. Figure 5A shows the CVs of the bare GCE, p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE, HPR-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE and CS/HPR-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE, respectively. Concerning the bare GCE (curve a, black dot line), no peak was observed as a result from the lack of redox-active material. After modified with the p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$) (curve b, red dash line), the redox peaks were observed due to the electrochemical behavior of an electrically conducting polymer [30-32]. According to Gao et al. [33], the current peaks decreased gradually after immobilization of HRP, and CS coating on the electrode surface, respectively. These results were attributed to the insulating property of HRP (curve c, green short dash line) and CS (curve d, blue solid line) (see Figure 5A).

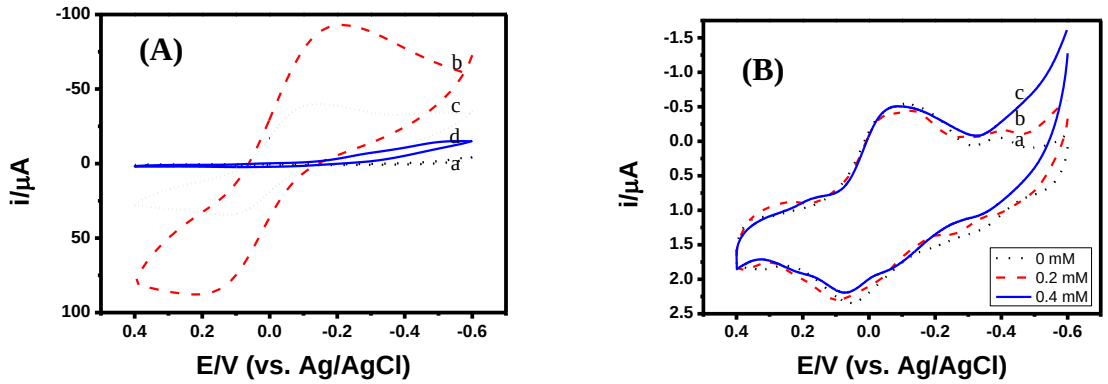

Figure 5. (A) CVs of (a) bare GCE (black dot line), (b) p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE (red dash line), (c) HPR-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE (green short dash line) and (d) CS/HPR-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE (blue solid line). (B) Typical CVs of the CS/HPR-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE in a N_2-saturated PBS (pH 6.5) in the absence (curve a, black dot line) and presence of 0.2 mM (curve b, red dash line) and 0.4 mM (curve c, blue solid line) of H_2O_2 using scan rate 50 mV/s at room temperature.

The typical cyclic voltammograms (CVs) of the CS/HPR-p(Ani$_{0.6}$-*co*-*o*-Aba$_{0.4}$)/GCE were investigated to determine the working potential for the electrochemical measurement of H_2O_2. The electrocatalysis of H_2O_2 reduction was studied by cycling the potential between +0.4 and -0.6 V (*vs.* Ag/AgCl) at the CS/HPR-p(Ani$_{0.6}$-*co*-*o*-Aba$_{0.4}$)/GCE to a Pt electrode in a PBS (pH 6.5) using scan rate of 50 mV s^{-1} at room temperature. As shown in Figure 5B, when 0.2 mM (curve b, red dash line) and 0.4 mM (curve c, blue solid line) of H_2O_2 were introduced into electrochemical system, the CS/HPR-p(Ani$_{0.6}$-*co*-*o*-Aba$_{0.4}$)/GCE showed an obviously electrocatalytic response to H_2O_2 (the reduction currents increased noticeably while the decrease of oxidation currents decreased). This feature implies that an enhanced enzymatic reduction of H_2O_2 was generated from the bioactivity of the immobilized HRP.

3.4 Electrocatalytic reaction mechanisms of CS/HRP-p(Ani-co-o-Aba)/GCE

The general features of the catalytic pathway for H_2O_2 reduction by HRP are currently well understood [16,34-36]. According to Berglund et al. [37], the electrocatalytic reaction mechanisms of the H_2O_2 biosensor based on the CS/HRP-p(Ani-*co*-*o*-Aba)/GCE could be schematically illustrated in Figure 6:

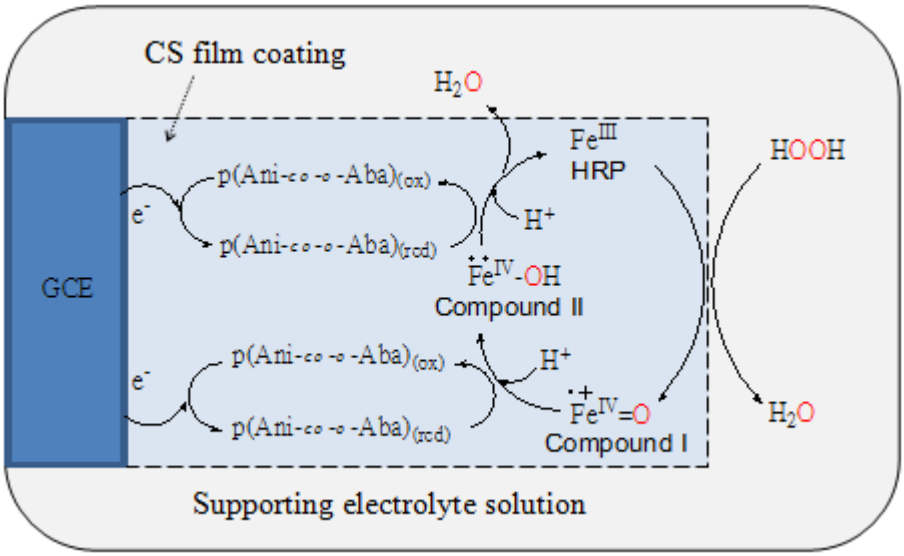

Figure 6. Electrocatalytic reaction mechanisms of the CS/HRP-p(Ani-*co*-*o*-Aba)/GCE in the presence of H_2O_2.

There are two different intermediates of horseradish peroxidase compounds that form during the reactions. They were created with either a reaction with hydrogen peroxide or an addition of an electron. Initially, the H_2O_2 in PBS diffused to the enzyme electrode, where it was enzymatically reduced by HRP (Fe^{III}) to give H_2O and to form the first intermediate (compound I). This intermediate is a two-equivalent oxidized form that contains an oxyferryl centre with the iron in the ferryl state

($^{+\bullet}Fe^{IV}$=O) and a porphyrin II cation radical. Then, the compound I shows the catalytic activity while its porphyrin radical undergoes the reduction to form the second intermediate (compound II, $^{\bullet\bullet}Fe^{IV}$-OH), which is subsequently regenerated to the native HRP by accepting one electron from the reduction state of copolymer, p(Ani-*co*-*o*-Aba)$_{(red)}$, on the electrode surface while copolymer itself changes into the oxidation state, poly(Ani-*co*-*o*-Aba)$_{(ox)}$. The poly(Ani-*co*-*o*-Aba)$_{(ox)}$ could be recycled to p(Ani-*co*-*o*-Aba)$_{(red)}$ at the electrode surface. When electrons were transferred from the electrode surface, the ampere currents were generated. Thus, the content of H_2O_2 could be determined by the measurement of currents. This approach was widely researched since it could be used to detect H_2O_2 with high sensitivity at a low quantitatively detectable concentration.

3.5 Optimization conditions for detection of H_2O_2 at CS/HRP-p(Ani-co-o-Aba)/GCE

The optimization conditions for the detection of H_2O_2 were examined by the CV method. In this work, the voltammetric currents of the novel biosensor could be controlled by changing the *o*-Aba mol ratio in p(Ani-*co*-*o*-Aba) synthesis, HRP concentration, CS concentration, pH and applied potential. The CS/HRP-p(Ani-*co*-*o*-Aba)/GCE was investigated by evaluating the reduction current to 0.2 mM H_2O_2 in a N_2-saturated PBS (pH 6.5).

The effect of *o*-Aba mol ratio in p(Ani-*co*-*o*-Aba) was varied from 0.2, 0.4, 0.6 and 0.8, respectively. The CS/HRP-p(Ani-*co*-*o*-Aba)/GCE prepared with the different *o*-Aba mol ratios was investigated by evaluating the reduction current to H_2O_2 at the applied potential of -0.3 V. As shown in Figure 7A, the current responses increased with increasing *o*-Aba mol ratio from 0.2 to 0.4 in the p(Ani-*co*-*o*-Aba), but decreased from 0.4 to 0.8. Therefore, a 0.4 mol ratio of the p(Ani-*co*-*o*-Aba) was selected for casting the modified electrode and for further experiments.

The effect of HRP concentration on the CS/HRP-p($Ani_{0.6}$-co-o-$Aba_{0.4}$)/GCE was studied in the presence of H_2O_2 at the potential of -0.3 V. As shown in Figure 7B, the current responses increased with increasing the HRP concentration to maximum value at 4 mg mL^{-1}, and then tended to decrease with further increase in the HRP concentration. This behavior is typical of the enzyme-based biosensors [35,38]. Thus, 4 mg mL^{-1} HRP was chosen for subsequent experiments.

The effect of CS concentration on the CS/HRP-p($Ani_{0.6}$-co-o-$Aba_{0.4}$)/GCE was studied in the presence of H_2O_2 at the potential of -0.3 V. The concentrations of CS solution were decreased from 1.0 to 0.2%wt. Li et al. [39] suggested that although high %CS solutions result in a high stability of modified electrode, high thickness of film coating also result in a large noise and slow response of biosensor. As seen in Figure 7C, the current responses increased with the decrease of %CS solution from 1.0 to 0.4%wt and then decreased from 0.4 to 0.2% wt. These results indicated that 0.2% wt of CS solution was not sufficient and would result in thin and unstable film on the modified electrode. Thus, to make a stable biosensor, a 0.4%wt of CS solution was selected for further investigations.

As known, the effect of pH on the H_2O_2 electrocatalytic biosensor is mainly due to the catalytic activity of the enzyme. In this work, the effect of pHs of a PBS on the current responses of H_2O_2 at the potential of -0.3 V was investigated from pH 5.0 to 8.0. According to Tangkuaram et al. [20], the acidic solution greatly enhanced the electrocatalysis of H_2O_2 by the HRP enzyme, because H^+ is

needed for HRP enzyme in the reduction of the H_2O_2 to H_2O. As shown in Figure 7D, the current responses increased rapidly from pH 5.0 to 6.5, and then slightly decreased from pH 6.5 to 8.0. Thus, to obtain the maximum sensitivity and bioactivity, the pH 6.5 of PBS was selected as the suitable pH of the supporting electrolyte solution for amperometric determination of H_2O_2.

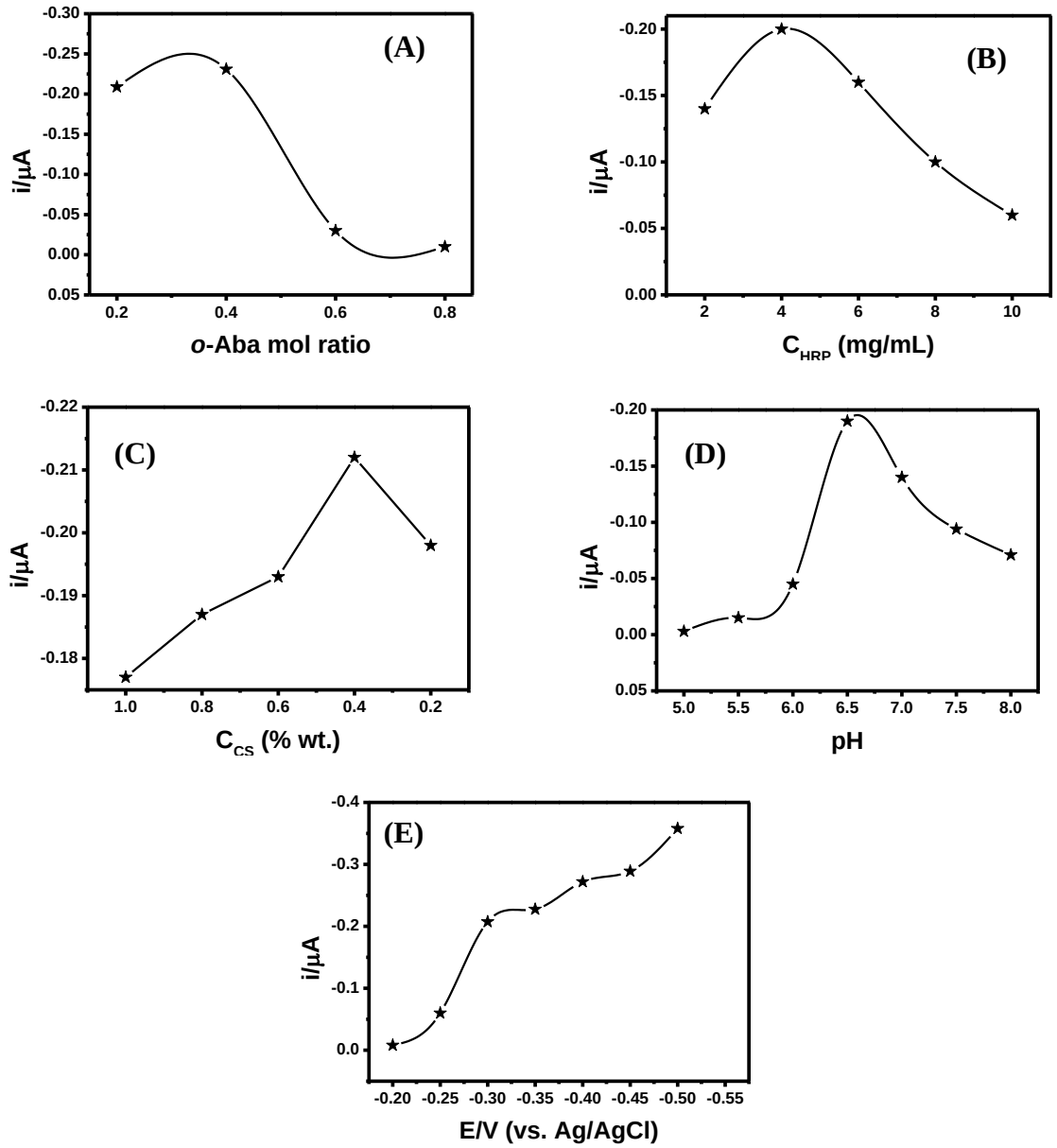

Figure 7. Dependence of current response to 0.2 mM H_2O_2 in a PBS at CS/HRP-p(Ani-*co*-*o*-Aba)/GCE: (A) *o*-Aba mol ratio, (B) HRP concentration, (C) CS concentration, (D) pH of PBS and (E) Applied potential.

The effect of applied potential on the current responses of H_2O_2 in a PBS (pH 6.5) using the CS/HRP-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE is investigated by evaluating the reduction current of H_2O_2 from -0.2 to -0.5 V. As shown in Figure 7E, the current responses increased when increasing the potential

from -0.2 to -0.5 V. Low current signal was observed below the potential of -0.3 V, but highly increased from -0.3 to -0.5 V. Although the CS/HRP-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE could have an excellent response at higher voltages, the high operating potential could result in interference from matrix species. Thus, a suitable applied potential of -0.3 V was selected for the determination of H_2O_2.

3.6 Amperometric measurement of H_2O_2 signal from CS/HRP-p($Ani_{0.6}$-co-o-$Aba_{0.4}$)/GCE

The amperometric measurement using the CS/HRP-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE was investigated by successively adding H_2O_2 to a continuous stirring 10 mL PBS solution under the optimized condition. Figure 8 shows the amperometric signals corresponding to its calibration plot under the optimal conditions. As shown in Figure 8A, the current increases with increasing the concentration of H_2O_2 ranging from 10 μM to 4 mM. The response time was very fast, indicating that the CS/HRP-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE had a higher sensitivity to H_2O_2. The calibration plot was constructed by plotting between the H_2O_2 concentration and the corresponding current. As shown in Figure 8B, the linear response range of the biosensor for H_2O_2 concentration was from 10 to 1,000 μM with a correlation coefficient (r^2) of 0.9905, and the detection limit of 1.8 μM was estimated based on the signal-to-noise ratio (S/N = 3).

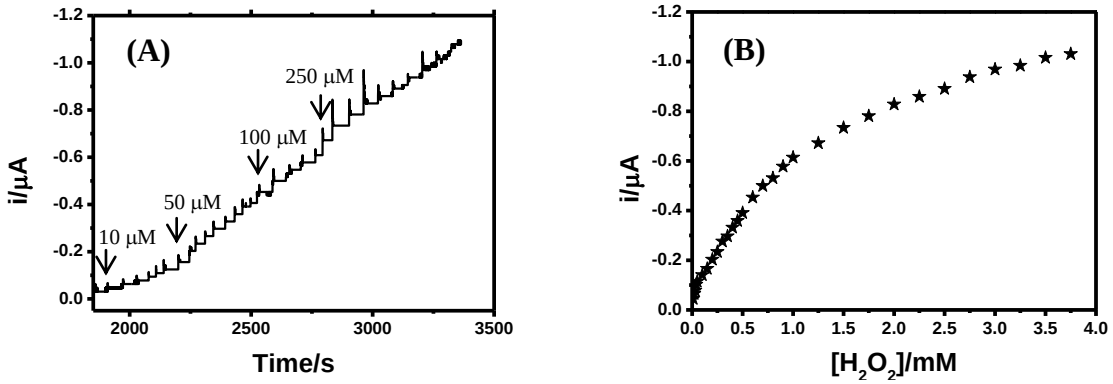

Figure 8. (A) Typical amperometic response of the CS/HRP-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE at the applied potential of -0.3 V (vs. Ag/AgCl) to the successive additions of H_2O_2 in a stirring PBS (pH 6.5). (B) The linear calibration plot of H_2O_2 concentrations *vs*. the corresponding current.

The apparent Michaelis-Menten constant (K_M^{app}), which gave an indication of enzyme-substrate kinetics, could be obtained from the electrochemical version of the Lineweaver-Burk equation:

$$\frac{1}{I_{ss}} = \frac{1}{I_{\max}} + \frac{K_M^{app}}{I_{\max} C}$$

where I_{ss} is the steady-state current after the addition of substrate, $I_{\max}$ is the maximum current under the saturated substrate condition, and C is the bulk concentration of the substrate. It was found

that the K_M^{app} value for the CS/HRP-p($Ani_{0.6}$-*co-o*-$Aba_{0.4}$)/GCE was calculated to be 5.14 mM, indicating that the CS/HRP-p($Ani_{0.6}$-*co-o*-$Aba_{0.4}$)/GCE was a high affinity of the enzyme immobilization for H_2O_2 electroanalytical determination. The K_M^{app} found here was smaller than such K_M^{app} value of 8.01 mM for the system of HRP-ZrO_2/Au electrode [40].

Table 1. Comparison of analytical performance of the proposed H_2O_2 biosensor towards H_2O_2 detection with previously reported modified electrodes.

Electrode	Applied potential (V)	Linear range (μM)	Detection limit (μM)	References
AuNPs-HRP/CS[a]	-0.3[d]	12.2-2,430	6.3	[41]
HPR/AuNPs/Thi/P(ABSA)[b]	-0.45[d]	2.6-8,800	0.64	[33]
HRP-ZrO_2[c]	-0.3[d]	20-9,450	2	[40]
HRP/PAni/CS[b]	-0.13[e]	10-1,500	0.5	[35]
SA/HRP-AuNPs[c]	-0.4[e]	20-13,700	3	[24]
HRP/PAni/MWCNTCOOH[c]	-0.35[e]	86-10,000	86	[42]
HRP/AuNPs-Thi/CS[b]	-0.38[e]	0.1-100	0.05	[43]
CS/HRP-p($Ani_{0.6}$-*co-o*-$Aba_{0.4}$)[b]	-0.3[e]	10 – 1,000	1.8	This work

AuNPs = gold nanoparticles; Thi = Thiophene; P(ABSA) = *p*-aminobenzene sulfonic acid; PAni = Polyaniline; MWCNTCOOH = Carboxy-functionalized multiwalled carbon nanotube; SA = Sodium alginate

[a] Carbon paste electrode (CPE)

[b] Glassy carbon electrode (GCE)

[c] Gold (Au) electrode

[d] Saturated calomel electrode (SCE)

[e] Silver/silver chloride (Ag/AgCl)

Table 1 shows the analytical performance of the proposed H_2O_2 biosensor towards H_2O_2 detection compared with various modified electrodes. This suggests that the linearity range, detection limit and response time of the proposed H_2O_2 biosensor mentioned above appeared to be beneficial to improve upon those of other previously reported modified electrodes.

3.7 Stability and selectivity of CS/HRP-p($Ani_{0.6}$-co-o-$Aba_{0.4}$)/GCE

Stability is important in practical use of the biosensors. In this study, the stability of the successive tests was investigated using the same biosensor and monitoring the current response of H_2O_2 at the applied potential -0.3 V (vs. Ag/AgCl). As shown in Figure 9A, it was found that the current responses of the biosensors gave the relatively closed results for the first 7 days, and the

electrochemical activity loss about 27% was observed after 13 days. These results suggested that the novel biosensor was a good satisfactory stability.

One of the most important challenges in the amperometric detection of H_2O_2 is the problem from other potential interferences. In the experiment, five interfering substances such as dopamine (DA), ascorbic acid (AA), glucose (Glu) and uric acid (UA) were used to evaluate the selectivity of the biosensor. Figure 9B shows a typical amperometic responses of the CS/HRP-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE at the applied potential -0.3 V (vs. Ag/AgCl) to the successive addition of the identical concentration of H_2O_2, DA, AA, Glu and UA in a continuously stirring PBS (pH 6.5). It was seen obviously that there was no significant change of the current responses generated from DA, AA, Glu and UA compared to the response of H_2O_2. Thus, these results strongly demonstrate that this modified electrode could be applied as the novel biosensor for a practical determination of H_2O_2 in the presence of AA, Glu, DA and UA.

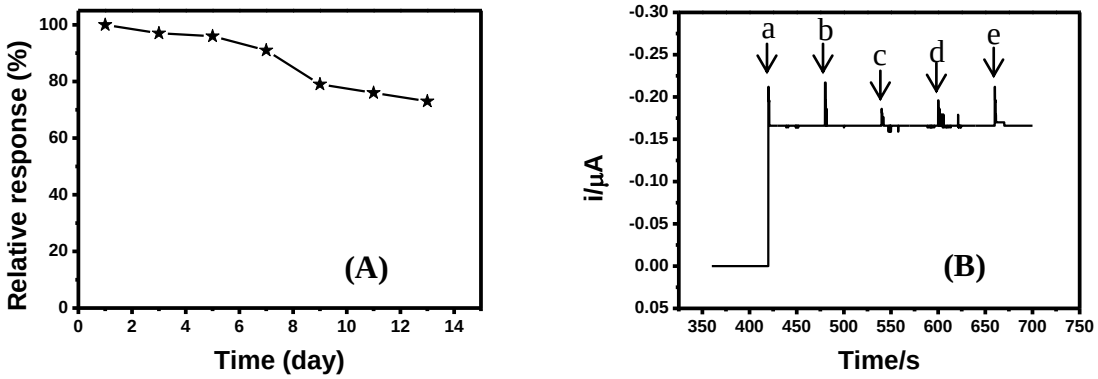

Figure 9. (A) Stability study of the CS/HRP-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE. (B) Amperometic responses of the CS/HRP-p($Ani_{0.6}$-*co*-*o*-$Aba_{0.4}$)/GCE at the applied potential of -0.3 V (vs. Ag/AgCl) to the successive addition of (a) 0.2 mM H_2O_2, (b) 1 mM DA, (c) 1 mM AA, (d) 1 mM Glu and (e) 1 mM UA in a stirring PBS (pH 6.5).

4. CONCLUSION

In conclusion, a novel method for fabrication of amperometric hydrogen peroxide biosensor was achieved by immobilizing horseradish peroxidase (HRP) on poly(aniline-*co*-*o*-aminobenzoic acid) (p(Ani-*co*-*o*-Aba)) through electrostatic attraction and then covered by chitosan (CS) film. A series of the p(Ani-*co*-*o*-Aba) having different compositions were prepared by the copolymerization of aniline (Ani) and *o*-aminobenzoic acid (*o*-Aba). The enzyme electrode exhibited a fast response toward H_2O_2, good linear range of response, as well as low detection limit. Based on these results, immobilizing HRP on p(Ani-*co*-*o*-Aba) through electrostatic attraction and then covered by CS film is useful strategy for preparing the amperometric enzyme biosensors.

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