Development of Hydrogen Peroxide Biosensor Based on Immobilization of Hemoglobin on Screen-Printed Carbon Electrode Modified With Silver Nanoparticles

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Abstract
The direct electron transfer behavior of hemoglobin that is immobilized onto screen-printed carbon electrode (SPCE) modified with silver nanoparticles (AgNPs) and chitosan (CS) was studied in this work. Cyclic voltammetry and spectrophotometry were used to characterize the hemoglobin (Hb) bioconjugation with AgNPs and CS. Results of the modified electrode showed quasi-reversible redox peaks with a formal potential of (-0.245V) versus Ag/AgCl in 0.1M phosphate buffer solution (PBS), pH 7, at a scan rate of 0.1Vs⁻¹. The charge transfer coefficient (α) was 0.48 and the apparent electron transfer rate constant (Ks) was 0.47s⁻¹. The electrode was used as a hydrogen peroxide biosensor with a linear response over 3 to 240 µM and a detection limit of 0.6 µM. As a result, the modified biosensor here has exhibited a high sensitivity, good reproducibility and stability.

Keywords: Hemoglobin, Silver Nanoparticles, Electrochemistry, Biosensor

Introduction
Hydrogen peroxide (H₂O₂) is one of the reactive oxygen species, which plays an important role in the regulation of a number of oxidative stress-related states. The rapid, selective and accurate

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estimation of H₂O₂ is crucial because it is an essential mediator in food, clinical, pharmaceutical and environmental researches [1]. Hydrogen peroxide is produced in several enzymatic reactions, therefore, it is important to detect different biological conditions [2]. Several sensitive analytical methods such as spectrometry, fluorimetry, tetrametry, chromatography and electrochemical techniques were developed to measure H₂O₂ concentration [3]. Among these, electrochemical techniques were extensively utilized due to low-cost, selectivity, simplicity, rapid response, high sensitivity and suitability for detecting analytics [4]. In electrochemical sensors, the direct detection of H₂O₂ at ordinary solid electrode is very limited because of high over-potential and slow electrode kinetics [5]. Therefore, the electrode is modified with proteins containing heme groups to improve its function.

Hemoglobin (Hb) is considered as a suitable model for H₂O₂ biosensor due to its peroxidase-like ability to reduce H₂O₂, its commercial availability with moderate cost and excellent stability, and its well-known structure [6]. Direct electron transfer between Hb and the conventional solid electrode is difficult because the distance between the active sites of the two is wide, whereas the active sites are deeply buried in polypeptide chains of the Hb molecule [7]. Different immobilization and modification procedures were studied to accelerate the electron transfer. Recently, nanoparticles attracted specific interests in the field of enzyme immobilization, due to unique properties such as high electrical conductivity, good chemical and thermal stability, and large surface area [8]. Silver, gold, and platinum are the most popular and widely used nanoparticles to develop Hb based H₂O₂ biosensor [9].

In the present study, the electrochemical behavior of Hb immobilized on screen-printed carbon electrode (SPCE) in the presence of silver nanoparticles (AgNPs) and chitosan (CS) was investigated and used to design as hydrogen peroxide biosensor. The designed biosensor was protected by Nafion (NF) molecules. In addition, the linearity, stability and reproducibility of NF-Hb-AgNPs-CS-SPCE were evaluated.

**Experimental part**

**Apparatus and measurement**

A potentiostat/ bipotentiostat (type DY2300, Digi-Ivy, Austin, U.S.A) was used to carry out the cyclic voltammetry, linear sweep voltammetry and amperometric measurements. A screen-printed carbon electrode (SPCE) from Digi –Ivy (USA) with three electrodes was employed, consisting of carbon electrode as working and counter electrodes, along with pseudo Ag/AgCl as a reference electrode. All the electrochemical experiments were performed at room temperature (28± 2 °C). The pH of the buffers was measured using a HANNA pH meter, whereas UV-Vis absorbance was studied using electronic spectra (UV-Visible; SHIMADZU 1800). In cyclic voltammetry (CV) and linear sweep voltammetry (LSV) measurement, 50µL of the analyte solution was dropped onto the reservoir area of the modified SPCE to cover the three electrodes, while successive addition (with stirring) of 10µL of H₂O₂ solution to a cell containing 30mL phosphate buffer solution (PBS; pH 7) was used for the amperometric measurement.

**Reagents**

Silver nanoparticles (0.02 mg/mL, suspension in aqueous buffer, 10nm), and Nafion (5%) were purchased from Sigma- Aldrich. Hb, and H₂O₂ from Sigma (USA), chitosan (CS) from shanghai Biochemical (China). A stock solution of 0.2M K₂HPO₄, KH₂PO₄ was used to prepare phosphate buffer solution (PBS, 0.1M) at various volume ratios and the pH was adjusted with 0.1M phosphoric acid or sodium hydroxide. All chemicals were of analytical reagent grade, and were used without further purification. Double distilled water (DDW) was used in all experiments. The stock solutions were stored at 4°C for further analysis.

**Preparation of the modified electrode**

The working electrode of SPCE was modified by washing with double distilled water (DDW) after pretreatment with (0.1 M) H₂SO₄ in a potential range of -0.5 V to + 1V at a scan rate of 0.1Vs⁻¹ for 25 cycles [10], then it was dried by nitrogen stream. Electro-deposition of CS on the working electrode was performed by dipping the SPCE in 2% chitosan solution (dissolved in 0.5N HCl) and a potential of -2 V was applied for 5 min. The resulted CS-SPCE was washed with DDW and dried at room temperature. Then, 15µL of the AgNPs (2µg/mL) were dropped onto the surface of the working electrode and allowed to dry at 4°C for 4 h.

For biomodification, 10µL of hemoglobin (6mg/mL) were drop by drop on the top of the modified SPCE which was allowed to dry in 4°C for 4 h. The modified SPCE was washed with DDW to remove
Results and Discussion

Characterization of NF-Hb-AgNps-CS UV-Vis spectra were collected to investigate the effect of chitosan and silver nanoparticles on the microstructures of Hb and conformations of heme group. As shown in Figure-1, the Hb was co-immobilized on AgNPs with CS and nafion (NF) assigned a sort band at 406nm, which was close to the sort band absorption of native Hb (405nm) (with higher absorbance intensity). This result could indicate that the co-immobilization process preserved the secondary structure of Hb and therefore no shift in the sort band occurred. It was reported that any blue shift of the sort band (or disappearance) of the Hb molecule could refer to Hb denaturation [11]. The spectrum of AgNPs (10nm diameter) showed a strong absorption at 408nm. Similar result was previously reported [12], which similar outcome of increased intensity of the sort absorption band of the heme iron in Hb-AgNPs-CS-system.

Direct electrochemistry of Hb in modified-SPCE

The CV responses of the bare and differently-modified electrodes were recorded using PBS (pH 7) with a potential range between - 0.6V to + 0.3V, and a scan rate of 0.1 Vs⁻¹. As shown in Figure-2, there was no obvious redox peak with bare-SPCE and AgNPs-CS-SCE, which could indicate that no redox substance was found on the surface of these electrodes. In the biomodification of the electrode with Hb molecules, different responses appeared with a pair of redox peaks, which could indicate that there was a direct electron transfer between Hb and the electrode.

Figure-2 shows a pair of small redox peaks for Hb- SPCE, which explains a slow electron transfer. However, the response was higher by around 2.6 times for NF-Hb-AgNPs-CS-SPCE. Thus, the presence of AgNPs was effective in the kinetics of electrode reaction through providing a suitable environment for better electron transfer between Hb and the underlying electrode.
The anodic and cathodic peak potentials were observed at -0.17 and -0.32V, respectively. The formal peak potential (E°) of Hb heme couple of Hb-AgNPs-CS-SPCE was -0.245V from (\[E° = \frac{Epa+Epc}{2}\]) \[13\], which was in the line with others \[14\]. The peak to peak separation (\[\Delta E_p\]) was 0.15 V and the anodic and cathodic peak currents were almost equal, which could imply that the Hb suffered a quasi-reversible electrochemical reaction. In addition, the apparent electroactive surface areas of bare and AgNPs-CS-SPCE were 0.04 and 0.07cm², respectively, when the CV was measured in 8mM Fe(CN)₆³⁻ with 0.1M KCl with a scan rate ranged from 0.01 to 0.5Vs⁻¹. This increase in the electroactive surface area perhaps resulted from the modification of SPCE with AgNPs. The influence of the potential scan on the electrochemical response of the immobilized Hb was studied in order to understand the behavior and reversibility of the electrode. Figure-3 shows the CV response obtained at NF-Hb-AgNPs-CS-SPCE in PBS (pH7) using different scan rates from 0.01 to 0.5Vs⁻¹. As the scan rate increased the anodic and cathodic peak currents increased. Moreover, no decrease in the redox peak current of Hb was found, although many repeated cycles were used, which could refer to strong immobilization of Hb molecules on the AgNPs.

![Graph showing CV of NF-Hb-AgNPs-CS-SPCE in 0.1M PBS (pH7) at different scan rates.](image)

**Figure 3**-The CV of NF-Hb-AgNPs-CS-SPCE in 0.1M PBS (pH7) at different scan rates (0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 Vs⁻¹).

A positive linear relation was determined between the oxidation and reduction peak currents of Hb with the scan rate, using the following regression equations:

\[Ipa = 26.242\nu +1.3179 \quad (R²=0.9955)\]

\[Ipc = -28.565\nu -1.4772 \quad (R²=0.9981).\]

Where Ipa is anodic peak potential, Ipc is cathodic peak potential and \(\nu\) is scan rate. The results implied that the redox behavior of Hb immobilized on the modified-SPCE was a surface-controlled quasi-reversible process \[15\]. According to the Randles-Scvcik’s equation \[16\]:

\[Ip = \frac{n²F²Aν}{4RT}\]

The peak current (Ip) is proportional to the number of electrons (n), surface area (A), scan rate (\(\nu\)), and surface coverage concentration (\(r\)). The slope in Figure-4 was used to calculate the electrochemical reaction value, which showed a single electron transfer, where \(r\) for Hb on the surface of the modified-SPCE was \(4.6×10^{10}\) mol. cm⁻². This \(r\) value was 20 times higher than the theoretical monolayer coverage (\(1.89×10^{10}\) mol. cm⁻²) \[17\]. High \(r\) value could indicate that AgNPs provided a large surface area and a three-dimensional architecture for hemoglobin immobilization, which increased the active sites for a better electron transfer process.

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The cathodic peak potential was plotted against the natural logarithm of the scan rate (lnυ), according to Laviron’s theory [18]:

\[ E_{pc} = E^o + \frac{RT}{\alpha n F} \ln \nu \]

where, Epc is the cathodic peak potential, E^o is the formal potential, T is the absolute temperature, R is the gas constant, υ is the scan rate, α is charge transfer coefficient and F is the Faraday constant.

The slope was used to calculate α value which was 0.48, thus, the redox reaction involved a single electron transfer. The heterogeneous electron transfer constant (Ks), which is an indicator to the electron transfer rate, was estimated according to the following Laviron’s equation:

\[ \ln K_s = \alpha \ln(1 - \alpha) + (1 - \alpha) \ln \alpha - \ln \frac{RT}{nF \nu} - \alpha (1 - \alpha) \frac{nF \Delta E_p}{RT} \]

Ks value here (0.478-1) was higher than that for Hb immobilized on other different surfaces. It was higher than that immobilized on Ag-AgO-silver electrode (0.239 s⁻¹) [19], and that on CNT-microelectrodes (0.062s⁻¹) [20]. However, it was similar to that of Hb on Au-cys-gold electrode (0.49s⁻¹) [21]. The result indicated that the electron transfer rate between Hb and modified-SPCE was very fast due to the presence of AgNPs, which promoted three-dimensional structures suitable for direct electron transfer of Hb towards the electrode surface. In this process, the distance between the redox center of Hb and the fabricated-SPCE was narrowed when compared with the distance between Hb and bare-SPCE [22]. Results in Figure-5 showed the effect of pH on the electrochemical behavior of Hb-modified electrode. A negative shift of the oxidation and reduction peak potential of immobilized Hb was indicated with an increased pH value from 3 to 8. The formal potential (E^o) of Hb was shifted linearly to a negative value when the pH increased from 3 to 8, with a linear regression E(V) of -0.043pH+0.054. This value was close to the theoretical expected value of -0.057V/ pH for one electron-one proton reaction [23]. These results suggest that there was one proton that collaborated in the electron transfer process. Furthermore, the highest peak current was observed at pH 7, where enough protons were available for the electrochemical reaction.
Electrochemical reduction of H$_2$O$_2$

The voltammetry response of NF-Hb-AgNPs-CS-SPCE was explored in PBS (pH 7) at a scan rate 0.1Vs$^{-1}$ in the absence and presence of 0.1 mM H$_2$O$_2$. As shown in Figure-6, upon the addition of H$_2$O$_2$, AgNPs-CS-SPCE did not provide any electrocatalytic activity towards the reduction of H$_2$O$_2$.

However, the NF-Hb-AgNPs-CS-SPCE affected the reduction and oxidation peak currents for H$_2$O$_2$. It enhanced the reduction peak current at a potential of -0.33 V by 1.8 times more than that for Hb-CS-SPCE, while it decreased the oxidation peak current. This could be explained by the assumption that the reduction process of H$_2$O$_2$ was typical for Hb. Alternatively, AgNPs were useful to provide effective adsorption sites for Hb molecules, which improved the catalysis process of H$_2$O$_2$ reduction by the heme group of Hb, where the active centers can be easily accessed, in addition to the increase in the electroactive area. Linear sweep voltammetry of modified electrode in different concentrations of H$_2$O$_2$ is shown in Figure-7. The cathodic peak current was increased with increased H$_2$O$_2$ concentration, and the current peak separation ($\Delta$I) reached a maximum value for each concentration of H$_2$O$_2$ at -0.33V. Hence, the potential of -0.33V was selected as the optimum monitoring potential in this study.
Figure 7- Linear sweep voltammetry (LSV) of the NF-Hb-AgNPs-CS-SPCE in PBS (pH 7) consists of different concentrations of H₂O₂.

A stable, well-defined and fast amperometric response of NF-Hb-AgNPs-CS-SPCE was obtained for successive addition of 20 µM H₂O₂ into constantly stirred 0.1M PBS (pH7). A percentage of 95 % of the maximum steady-state current was achieved within less than 10s after each injection of the analytic solution.

The current time response of the modified SPCE was explored in H₂O₂ concentration ranging 0 - 300µM. Figure-8 shows that the reduction peak current of H₂O₂ for the biosensor under study was linear over a concentration range of 3 to 240 µM, and better than the previous biosensors (10-120 µM and 10-150 µM) [6, 24].

Figure 8-Amperometric response of NF-Hb-AgNPs-CS-SPCE with successive addition of 20 µM of H₂O₂ in 0.1M PBS (pH 7) at a potential of -0.33V.

To illustrate the ability of the biosensor to calculate the low concentration of H₂O₂, the detection limit was calculated to be 0.6 µM with a signal-to-noise ratio of around three (S/N=3), which was lower/ better than that reported previously [25]. The sensitivity of biosensor (0.57 µA. µM⁻¹.cm²) was calculated from the slope, where it was higher than that for Hb-ZrO₂-collagen-GCE (0.0456 µA. µM⁻¹.cm²) [26]. The Michaelis-Menten constant (Kₘ) of hemoglobin toward H₂O₂ in the present biosensor can be obtained from the electrochemical version of the Line weaver-Burk equation [27]:

\[
y = \frac{0.04x + 0.9002}{R^2 = 0.9987}
\]
\[
\frac{1}{I_{ss}} = \frac{1}{I_{\text{max}}} + \frac{K_{\text{app}}}{I_{\text{max}} \times C}
\]

Where, \( C \) is the bulk concentration of the substrate (\( \text{H}_2\text{O}_2 \) in this study), \( I_{ss} \) is the steady-state current after the addition of the substrate, and \( I_{\text{max}} \) is the maximum current measured under saturated substrate conditions. The calculated value of \( K_{\text{app}} \) was 0.315\( \mu \text{M} \), which was smaller than previously reported values for \( \text{H}_2\text{O}_2 \) biosensor (77.7\( \mu \text{M} \) and 45.35\( \mu \text{M} \)) [6, 28]. A low \( K_m \) value is well reflected in a high affinity toward the substrate [29]. Herein, the immobilized \( \text{Hb} \) has a high affinity for \( \text{H}_2\text{O}_2 \) electrocatalytic reduction. The repeatability of the modified electrode was evaluated by the CV method. The relative standard deviation (R.S.D) values were 3.1\% and 4.2\% for 20 successive measurements of 0.1 and 0.2\( \text{mM} \) \( \text{H}_2\text{O}_2 \), respectively. While, the reproducibility of the biosensor was evaluated by measuring the CV of 0.1\( \text{mM} \) \( \text{H}_2\text{O}_2 \) in six different modified electrodes, prepared independently. The results showed an acceptable reproducibility with an R.S.D of 3.4\%.

The long-term stability of the present biosensor was studied at \( 4^\circ \text{C} \) over a course of 30 days and the response was tested every 5 days. The results indicated that the electrodes lost 3.1\% of the initial activity after 10 days, 7\% after 20 days and the maximum loss was 11.2\% over 30 days. Accordingly, the repeatability, reproducibility and long-term stability could be attributed to the high chemical stability and good biocompatibility of AgNPs, which caused a tight immobilization of \( \text{Hb} \) molecules onto the surface of the modified electrode.

Conclusion

This study aimed to prepare a stable and reproducible \( \text{H}_2\text{O}_2 \) biosensor modified with nanoparticles. The \( \text{Hb} \) molecules was efficiently immobilized onto a screen-printed carbon electrode modified with silver nanoparticles and chitosan. It was protected by coating with 0.5\% nafion solution. The results showed that silver nanoparticles enhanced the direct electron transfer between \( \text{Hb} \) and the electrode due to its high surface area. The resulted biosensor was used to determine \( \text{H}_2\text{O}_2 \) concentration over a range from 3 to 240\( \mu \text{M} \), with a low detection limit. The modified biosensor prepared here showed a long-term stability, good reproducibility and repeatability.

References


