Direct Electrochemistry of Hemoglobin Immobilized on a Functionalized Multi-Walled Carbon Nanotubes and Gold Nanoparticles Nanocomplex-Modified Glassy Carbon Electrode

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Abstract: Direct electron transfer of hemoglobin (Hb) was realized by immobilizing Hb on a carboxyl functionalized multi-walled carbon nanotubes (FMWCNTs) and gold nanoparticles (AuNPs) nanocomplex-modified glassy carbon electrode. The ultraviolet-visible absorption spectrometry (UV-Vis), transmission electron microscopy (TEM) and Fourier transform infrared (FTIR) methods were utilized for additional characterization of the AuNPs and FMWCNTs. The cyclic voltammogram of the modified electrode has a pair of well-defined quasi-reversible redox peaks with a formal potential of $\pm 0.270 \pm 0.002$ V (vs. Ag/AgCl) at a scan rate of 0.05 V/s. The heterogeneous electron transfer constant (ks) was evaluated to be $4.0 \pm 0.2$ s$^{-1}$. The average surface concentration of electro-active Hb on the surface of the modified glassy carbon electrode was calculated to be $6.8 \pm 0.3 \times 10^{-10}$ mol cm$^{-2}$. The cathodic peak current of the modified electrode increased linearly with increasing concentration of hydrogen peroxide (from 0.05 nM to 1 nM) with a detection limit of 0.05 ± 0.01 nM. The apparent Michaelis-Menten constant ($K_{m}^{app}$) was
calculated to be 0.85 ± 0.1 nM. Thus, the modified electrode could be applied as a third generation biosensor with high sensitivity, long-term stability and low detection limit.

**Keywords:** hemoglobin; direct electrochemistry; functionalized multi-walled carbon nanotubes; gold nanoparticles; nanocomplex

1. Introduction

Direct electrochemistry of redox proteins immobilized on different electrodes has recently attracted great attention. These methods can provide a suitable model for understanding the electron transfer mechanisms in biological systems and to establish a foundation for fabrication of electrochemical biosensors and devices [1–4]. Successful approaches have included cast films of redox proteins with different materials and membranes [5–23]. Achieving direct electron transfer between redox proteins or enzymes and electrodes simplifies these devices, which has a great significance in preparing the third generation of biosensors [24].

Hemoglobin (Hb) is an oxygen carrier and as a pro-oxidant takes part in complex redox processes in the blood. This protein consists of two alpha and two beta subunits with a molecular weight of about 67,000 and each subunit has one peptide chain and one protoheme [25–27]. Hb structure is similar to the peroxidases and can catalyze the reduction of hydrogen peroxide. [28,29]. Direct electron transfer of Hb immobilized on various electrode materials has become popular. In addition, the structure of Hb is known and can be used as a model to explain the relationship between the protein structure and function and to construct new functional biosensors without mediators [13,30–32].

Carbon nanotubes are widely used for the fabrication of electrochemical biosensors, because of their special structure and properties such as high surface area, which makes them a suitable material to transfer electrons [33–35]. Gold nanoparticles are one of the most stable metal nanoparticles, and have been widely applied in analytical chemistry and electrochemistry, because of their novel optical, electrical and catalytic properties and favorable biocompatibility [36,37]. Each type of nano-material has its own physical and chemical characteristics, which makes the design and preparation of biosensors based on only one type of nanomaterial laborious. Thus, the use of composites from several type of materials could be preferable [38]. Recently, several nano materials, including carbon nanotubes and nanoparticles of Au, Ag, TiO$_2$, Fe$_3$O$_4$ or MnO$_2$, have been applied in electrochemical studies of hemoglobin and other redox proteins [39]. Immobilization of redox proteins on nanocomplexes is a new way to realize their direct electrochemistry.

In our previous study, a nanocomplex consisting of carboxylic acid functionalized multi-walled carbon nanotubes (FMWCNTs) and gold nanoparticles (AuPNs) was modified on a glassy carbon (GC) electrode, and applied to analysis the electrochemical properties of catalase [40] and heme-containing artificial peroxidase [41].

In the present study, direct electron transfer of Hb was realized when it was immobilized on the nanocomplex-modified glassy carbon (GC) electrode. Thus, this electrode could be used as a high sensitivity hydrogen peroxide (H$_2$O$_2$) biosensor.
2. Experimental Section

2.1. Chemicals

Hb from bovine erythrocyte, L-cysteine (Cys), NF (5%), HAuCl₄ and sodium citrate were from Sigma (Saint Louis, MO, USA) and used without further purification. Multi-wall carbon nanotubes (MWCNTs), prepared by chemical vapor deposition, were purchased from Shenzhen Nanotech Port Ltd. Co. (Shenzhen, China). Hydrogen peroxide, sodium dihydrogen phosphate (NaH₂PO₄) and disodium hydrogen phosphate (Na₂HPO₄) were obtained from Shanghai Chemicals Company (Shanghai, China). All solutions were prepared in double-distilled deionized water. The stock solutions of hydrogen peroxide (H₂O₂) were prepared by appropriate dilutions of 30% (v/v) H₂O₂ in deionized water. All other chemicals were of analytical grade and used without further purification.

2.2. Preparation of Gold Nanoparticles (AuNPs)

AuNPs were prepared as previously reported in the literature [42–44]. Briefly, 0.01% HAuCl₄ solution was heated, then 0.02 M sodium citrate solution was dropped quickly into the hot HAuCl₄ solution while agitating vigorously. At first, the color of solution changed from light yellow to grey, then to black and gradually to wine red color without further change. At this stage, the color no longer changed, the heating was stopped, and the solution mixture was continuously agitated until it was cooled to room temperature. The prepared gold colloidal nanoparticles (AuNPs) were stored in dark at 4 °C.

2.3. Preparation of Functional Multi-Walled Carbon Nanotubes (FMWCNTs)

MWCNTs were functionalized according to published methods [23]. Briefly, purified MWCNTs were treated with a concentrated mixture of H₂SO₄ and HNO₃ (v/v = 1/3) under supersonic bath condition (KQ-100B Supersonic Cleaner, Kunshan Shumei, Kunshan, China) for 4 h at 80 °C to introduce carboxyl groups on their surface. The solution pH was then adjusted to 7 with 1 M NaOH solution, centrifuged and washed with water three times. The obtained MWCNTs-COOH (FMWCNTs) were dried at room temperature.

2.4. Preparation of Functional Membrane Modified Glassy Carbon (GC) Electrode

The preparation of the GC electrode was as previously described [45–49]. Prior to coating, the GC electrode was mechanically polished twice with alumina (particle sizes 1.00, 0.30 and 0.05 μm, respectively) to a mirror finish. The electrode was then treated electrochemically in 0.2 M sulfuric acid, cycling between −1.0 and +0.5 V (vs. Ag/AgCl) at a sweep rate of 0.1 V/s for approximately 10 min. Thereafter, the working electrode was placed in a 50 mM PBS (pH 7.0), and an anodic potential of 1.70 V (vs. Ag/AgCl) was applied for 3–5 min. After the electrode was washed, the AuNPs (negative charged) were electro-deposited on a cleaned bare GC electrode in the range of 0.0 to 1.1 V 25 cycles at a scan rate of 0.1 V/s [50]. The GC electrode was then dipped in 1.0 mM L-cysteine (Cys) for 30 min, washed with water, 3 μL of FMWCNTs (2 mg/mL) was dropped onto the surface of the electrode, and dried at room temperature. The electrode was dipped in a Hb solution (80 μM) for 24 h.
at 4 °C, and for protection, 2 μL Nafion (NF, 5%) was dropped on the electrode surface. The preparation process of functional membrane modified glassy carbon (GC) electrode was also shown in Figure 1.

**Figure 1.** Preparation process of functional membrane modified glassy carbon (GC) electrode.

2.5. Apparatus and Measurements

Electrochemical studies were carried out in a conventional three-electrode cell powered by an electrochemical system comprising of CHI650C (CHI Instruments, Austin, TX, USA). An Ag/AgCl-saturated KCl, a Pt wire and a GC electrode of 3 mm diameter (CHI Instruments) were used as the reference, counter and working electrodes, respectively. All of the potentials in this article were with respect to Ag/AgCl. The electrochemical measurements were carried out in N₂-saturated 0.05 M sodium phosphate buffer solution (PBS) at pH 7.0, 20 °C. Electron microscopic images (TEM) of FMWCNT and AuNPs were obtained using a JEM-1400 (JEOL, Musashino, Japan). Fourier transform infrared (FTIR) spectra of FMWCNTs by KBr pellets were collected in the range of 1,000–3,500 cm⁻¹ on a FTIR 4300 (Shimadzu, city, Japan) spectrometer at room temperature. UV-vis absorption spectra of the AuNPs were collected using a TU-1901 spectrophotometer (Beijing Purkinje General Instrument Company, Beijing, China), with 1 cm path length cells equipped with a thermostat holder and an external temperature controller (Shanghai Hengping Instrument Company, Shanghai, China) at 25 ± 0.1 °C.

3. Results and Discussion

3.1. Characteristics of the Nanomaterials

The AuPNs and FMWCNTs were characterized by TEM. 3 μL of AuPNs (0.254 mM) or FMWCNTs (3 mg/mL) was dropped onto the surface of fomvar/carbon coated grids (300 mesh), dried and then viewed by TEM operating at 80 kV [40], respectively. UV-vis spectroscopy of the prepared AuNPs exhibited a maximum absorption at 522 nm. The mean size of the AuNPs was then determined to be 18.4 ± 1.1 nm [40,51]. The FTIR spectrum of FMWCNTs shows the characteristic peaks at 1,709, 1,172, and 3,402 cm⁻¹ correspond to the C=O, C–O and O–H stretching vibration of the carboxyl group [52–54], respectively, which indicates that carboxyl groups were modified on the MWCNTs (data not shown).
3.2. Electrochemical Studies

Figure 2(A) presents the cyclic voltammograms (CVs) of: (a) bare electrode; (b) NF/Hb/GC electrode; (c) NF/FMWCNTs/Cys/AuNPs /GC electrode; (d) NF/Hb/FMWCNTs/GC electrode; and (e) NF/Hb/FMWCNTs/Cys/AuNPs/GC electrode at a scan rate of 0.05 V/s. It can be seen that either (d) or (e) show a well-defined redox wave. The electrode (e) had a stronger redox peak current than that of (d), and the AuPNs could help to significantly increase the redox peaks. Moreover, cathodic and anodic peak potentials were −0.309 V and −0.231 V (vs. Ag/AgCl), respectively. Thus, the formal potential (E° = (Epa + EpC)/2) of the electrode (e) was −0.270 ± 0.002 V (vs. Ag/AgCl). This value is consistent with the E° obtained for an Hb/PLGA/ILs/GC electrode (−0.318 V vs. SCE) [21], an Hb/BMS/CS/GC electrode (−0.32 V vs. Ag/AgCl) [55], or an Hb/Chit-[bmim]PF6-TiO2-Gr/GC electrode (−0.206 V vs. SCE) [17], which is the characteristics of the Hb heme Fe(III)/Fe(II) redox couple. It is notable that the positive potential for the electrode facilitated the electrode reaction (Equation (1)) and led to a more efficient bio-catalytic reduction [46,47,49]:

\[ k_p = Ae^{-\Delta G/RT} e^{-\alpha n FE}. \]  

The separation between the anodic and cathodic peak potential (Ep = Epa - EpC = 78 mV), and the current ratio of the anodic peak current to the cathodic one (Ipa/Ipc ≈ 1), indicate that the electrochemical process of the modified GC electrode (e) is quasi-reversible [46,47]. Thus, AuNPs can greatly improve the redox current-modified electrode and facilitate a fast direct electron transfer of Hb entrapped in the composite film. In addition, it is worth noting that there was a pair of unstable redox peaks for curves at site 1 and 2, which should be due to Cys-modified Au, and this redox peak would disappear gradually while the electrode was working. CVs of NF/Hb/FMWCNTs/Cys/AuNPs/GC electrode in 50 mM PBS (pH 7.0) at various scan rates are shown in Figure 2(B). The peak currents increased with increasing the scan rate (v) and were linearly proportional to v (Figure 2C) (not v^1/2). The linear regression equations for cathodic (Ipc) and anodic peak (Ipa) currents are: Ipc = 12.65v + 0.14 and Ipa = −12.45v − 0.13, with the correlation coefficients of 0.9981 and 0.9977, respectively. The CVs remained essentially unchanged on consecutive potential cycling, indicating that modified electrode is stably confined on the glassy carbon electrode. Figure 2(D) shows the relationship between the peak potential (Ep) and the natural logarithm of scan rate (lnv) for the modified electrode. In the range from 1.2 to 4 V/s, the cathodic peak potential (EpC) changed linearly vs. lnv with a linear regression equation of EpC = −0.0302 ln(v) − 0.324, r = 0.991. According to Laviron’s Equation [56]:

\[ E_p = E^° + \frac{RT}{anF} - \frac{RT}{anF} \ln v \]  

where α is the cathodic electron transfer coefficient, n is the number of electrons, T is the temperature (293 K here), R the gas constant (8.314 JK⁻¹mol⁻¹) and F the Faraday constant (96,485 C mol⁻¹), respectively. RT/anF was 0.0302 here, then, α could be calculated to be 0.55. It could be concluded that n = 1 and α = 0.55 ± 0.05 [57]. The n = 1 can be also obtained from the width of the peak at mid-height with a low scan rate. Thus, the redox reaction between Hb and the glassy carbon electrode is a single electron transfer process.
Figure 2. (A) CVs of different modified electrodes (from inner to outer): (a) Bare GC electrode; (b) NF/Hb/GC; (c) NF/FMWCNTs/Cys/AuNPs/GC; (d) NF/Hb/FMWCNTs/GC; (e) NF/Hb/FMWCNTs/Cys/AuNPs/GC. The experiments were carried out in 0.05 M PBS (pH 7.0) at a scan rate of 0.05 V/s. (B) CVs of NF/Hb/FMWCNTs/Cys/AuNPs/GC electrode in 0.05 M PBS (pH 7.0) at various scan rates (from inner to outer): 0.02, 0.04, 0.06, 0.08, 0.1, 0.12, 0.14, 0.16, 0.18... 4 V/s, respectively; (C) Plot of peak current $I_p$ vs. scan rate $\nu$; (D) Plot of peak potential $E_p$ vs. $\ln(\nu)$.

The value of the apparent heterogeneous electron transfer rate constant $k_s$ could be calculated using the following equation based on Laviron’s Equation [58]:

$$\ln k_s = \alpha \ln(1 - \alpha) + (1 - \alpha) \ln(\frac{RT}{nFv}) - \alpha(1 - \alpha) \frac{nF\Delta E_p}{RT}. $$

(3)

Then, $k_s$ was calculated to be $4.0 \pm 0.2$ s$^{-1}$. This value was higher than the most reported $k_s$ values of Hb immobilized on GC electrodes [5,9,10,17,23], due to the high specific surface area and good biocompatibility of the nano-complex.

The average surface concentration ($\Gamma$) of electro-active sites (heme groups) of Hb on the surface of glassy carbon electrode could be estimated based on the slope of $I_p$ vs. $\nu$ (Equation (4)) [59]:

$$I_p = \frac{n^2F^2\Gamma \nu}{4RT}. $$

(4)
The value of $\Gamma$ was calculated to be $6.8 \pm 0.3 \times 10^{-10}$ mol·cm$^{-2}$, which is higher than the $\Gamma$ value of monolayer of Hb $1.89 \times 10^{-11}$ mol·cm$^{-2}$ [60,61]. This high surface concentration can be attributed to the AuNPs and FMWCNTs nanocomplex. The larger surface area and good biocompatibility of the nanocomplex may be helpful for more efficient Hb entrapped in the nanocomplex membrane and provides more activity sites to take part in the electron transfer process (see also Figure 1).

3.3. pH Effect

Figure 3(A) represents CVs of the modified electrode in 50 mM PBS at various pH values. An increase in pH of the solution from 5.0 to 8.0 led to a negative shift in both reduction and oxidation peak potentials. Figure 3(B) shows that the cathodic peak’s current increased with pH changes from 5.0 to 7.0, and then decreased when the pH was greater than 7.0. The maximum cathodic current was obtained at pH 7.0. Figure 3(C) shows that the formal potential of the electrode is pH dependent. These results indicated that the slope was $28.1 \pm 0.4$ mV/pH over a pH range of 5.0 to 8.0. This value was much smaller than the ideal Nernst’s value of 59.2 mV/pH for a one electron and one proton process [59]. The reason might be the biocompatible micro-environment provided by NF. This makes the electrode more stable to pH changes [45–48], influences the protonation state of trans ligands to the heme iron and amino acids around the heme, or the protonation of the water molecules coordinated to the central iron [62].

Figure 3. (A) CVs of NF/Hb/ FMWCNTs/Cys/AuNPs/GC electrode in 0.05 M PBS at different pH values: (a) 5.0, (b) 6.0, (c) 7.0, and (d) 8.0, respectively; (B) plot of $I_{pc}$ vs. pH value; (C) Plot of $E^{\circ}$ vs. pH value.

\[ y = -0.0281x + 0.0051 \]
\[ R^2 = 0.9932 \]
3.4. Optimum Monitoring Potential

Figure 4(A) shows the linear sweep voltammograms (LSVs) of NF/Hb/FMWCNTs/Cys/AuNPs/GC electrode in the absence (a) and presence of H$_2$O$_2$ and with 0.1, 0.13, 0.16, 0.2 mM H$_2$O$_2$ (b–e). The cathodic peak current increased with increased concentration of H$_2$O$_2$. Thus, the electrode is sensitive to the tested concentrations of the chosen substrate and used as a biosensor of H$_2$O$_2$. In addition, the ΔI reached a maximum value for each concentration of H$_2$O$_2$ at about −350 mV. Hence, the potential of −350 mV (vs. Ag/AgCl) was selected as the optimized monitoring potential throughout this study.

It is worth noting that either Cys/AuNPs/GC (Figure 4(B)) or NF/Cys/AuNPs/GC (Figure 4(C)) electrodes exhibited an amperometric response to H$_2$O$_2$ at potentials higher than −0.6 V (vs. Ag/AgCl). These results were similar to those reported in the literature [63,64]. However, Figure 4(D) shows that the amperometric response of the NF/Hb/AuNPs/GC electrode toward H$_2$O$_2$ occurred at an even lower potential (−400 mV) with stronger current intensity. Thus, these results could be attributed to the bioelectro-catalytic behavior of Hb.

Figure 4. LSVs of (A) NF/Hb/FMWCNTs/Cys/AuNPs/GC electrode in the absence or presence of different concentrations of H$_2$O$_2$ (from curve a to curve e): 0, 0.1, 0.13, 0.16, 0.20 mM, respectively. LSVs of (B) Cys/AuNPs/GC; (C) NF/Cys/AuNPs/GC and (D) NF/Hb/Cys/AuNPs/GC electrodes, respectively in the presence of different concentrations of H$_2$O$_2$ (from a to curve d): 0.1, 0.3, 0.5 and 0.7 mM, respectively.
3.5. Electro-Catalytic Behavior of Modified Electrode and Detection Limit

Figure 5 presents the current responses of the modified electrode to successive additions of 5 μL of 1 nM (a), 10 nM (b) or 20 nM (c) of H$_2$O$_2$ in 5 mL of 0.05 M PBS (pH 7.0) at the applied potential of −0.35 V (vs. Ag/AgCl). The inset shows the typical current response for each addition process. Current at state 1 is a steady state ($I_{sa}$) with no addition, when H$_2$O$_2$ is added the current increases rapidly and reaches a maximum, state 2 ($I_{ma}$). The current then reduces gradually to a steady state, state 3 ($I_{sb}$), before the next addition and next maximum current response ($I_{mb}$), state 4. Though the amount of maximum response current was great, this state was unsuitable for use, as the change of the maximum current response ($ΔI = I_{mb} - I_{ma}$) was not stable for each addition, and was affected by the mainly addition position and diffusion rate of H$_2$O$_2$. The steady state current response ($ΔI = I_{sb} - I_{sa}$) increased when the addition concentration of added H$_2$O$_2$ was over 10 nM with the final concentration of H$_2$O$_2$ (0.01–50 nM) and a linear range from 0.05 to 1 nM (see also Figure 6(A)).

![Figure 5](image-url)

Figure 5. Amperometric response of the modified electrode to successive additions of 5 μL of 1 nM (a), 10 nM(b) or 20 nM(c) H$_2$O$_2$ in 5 mL of 0.05 M PBS, pH 7.0, at the applied potential of −0.35 V (vs. Ag/AgCl). Inset shows the typical current response for each addition process: (1) previous steady state current; (2) maximum response current; (3) steady state current; (4) next maximum response current.

To determine the detection limit (minimum concentration of H$_2$O$_2$ that could be detected by this method), the steady current ($I_s$) of each addition was measured while the final concentration of H$_2$O$_2$ ([H$_2$O$_2$]) was increased gradually (Figure 6(B)). The detection limit was determined to be 0.05 ± 0.01 nM from the cross point of the lines fitted to the linear segments of the $I_s$ vs. [H$_2$O$_2$] [65,66], which was lower than the most reported values, e.g., an Hb/GNPs/Hb/MWNT/GC electrode (80 nM) [67] and a CAT/[bmim][PF$_6$]/MWCNTs/GC electrode (0.25 nM) [68].
Figure 6. (A) The typical steady current \(\text{vs.} [\text{H}_2\text{O}_2]\) in the process of successive additions of 5 \(\mu\)L of 10 nM (●) and 20 nM (▲) \(\text{H}_2\text{O}_2\) in 5 mL of 50 mM PBS (pH 7.0) at the applied potential of \(-0.35\) V (vs. Ag/AgCl). (B) The determination of the \(\text{H}_2\text{O}_2\) detection limit for NF/Hb/FMWCNTs/Cys/AuNPs/GC electrode. The detection limit was determined from the cross point of the lines fitted to the linear segments of the steady current \(I_s\) \(\text{vs.} [\text{H}_2\text{O}_2]\) in the process of successive additions of 5 \(\mu\)L of 10 nM \(\text{H}_2\text{O}_2\) in 5 mL of 50 mM PBS (pH7.0). (C) Lineweaver-Burk plot for \(K_{\text{m}}^\text{app}\) determination.

3.6. Kinetic Parameters

Overall, the electrode reaction could be supposed to be carried out by two steps [40]:

Step One: \(\text{Hb(Fe}^{\text{III}}\) + e + \(H^+\) = \(\text{Hb(Fe}^{\text{II}}-\text{H})\)

Step Two: \(\text{Hb(Fe}^{\text{II}}-\text{H}) + \frac{1}{2} \text{H}_2\text{O}_2 \rightarrow \text{Hb(Fe}^{\text{III}}\) + \(H_2O\)

where, \(\text{Hb(Fe}^{\text{III}}\) and \(\text{Hb(Fe}^{\text{II}}-\text{H})\) denote the oxidized and reduced forms of the modified Hb, respectively. Initially \(\text{Hb(Fe}^{\text{III}}\) undergoes the electron transfer reaction with the electrode resulting in the production of \(\text{Hb(Fe}^{\text{II}}-\text{H})\), as shown in Step One, this is an one-electron and one-proton (pH dependent) process. Alternatively, \(\text{Hb(Fe}^{\text{II}}-\text{H})\) can be oxidized by \(\text{H}_2\text{O}_2\) in the solution to regenerate \(\text{Hb(Fe}^{\text{III}}\) as shown in Step Two. In this catalytic reaction, the peak current kept rising until \(\text{Hb(Fe}^{\text{III}}\) was consumed in the Step One and was compensated by its production in Step Two.
The apparent Michaelis-Menten constant $K_{m\text{app}}$ is a reflection of both enzyme affinity and ratio of microscopic kinetic constants. It can be obtained from the electrochemical version of the Lineweaver-Burk Equation [13]:

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_{m\text{app}}}{I_{max}c}$$  \hspace{1cm} (5)

where, $c$ is the substrate (H$_2$O$_2$) concentration in the solution ([H$_2$O$_2$] here), $I_{ss}$ the steady-state current after the addition of substrate, and $I_{max}$ is the maximum current measured under saturated substrate conditions. $K_{m\text{app}}$ value of the modified electrode was calculated to be 0.85 ± 0.1 nM (Figure 6(C)), which was much lower than the most reported values, e.g., an HRP-AQ/GC electrode (51 nM) [49], a PLGA/ILs/Hb/GC electrode (69 μM) [21], and an Hb/Chit-[bmim]PF$_6$-TiO$_2$-Gr/GC electrode (1.245 mM) [17]. A low $K_{m\text{app}}$ value indicates a strong substrate binding and exhibits a higher affinity of H$_2$O$_2$ for this modified electrode. FMWCNTs and AuNPs complex system helps to reduce the bridge length between electroactive center (heme group) of Hb and GC electrode [47], and result in the high sensitivity of the modified electrode to H$_2$O$_2$ (Table 1).

**Table 1.** Comparison of electrochemical parameters of Hb on different modified GC electrodes.

| Modified Electrode | $E^*$ (mV) | $k_0$ (s$^{-1}$) | $\Gamma$ (mol·cm$^{-2}$) | $K_{m\text{app}}$ | Linear Range | Detection Limit | Ref |
|--------------------|------------|-----------------|--------------------------|------------------|--------------|-----------------|-----|
| NF/Hb/FMWCNTs/Cys/AuNPs/GC | $-270 \pm 2^a$ | 4.0 ± 0.2 | $6.8 \pm 0.3 \times 10^{-10}$ | 0.85 nM | 0.05–1 nM | 0.05 nM | This work |
| Hb/PdNPs/GR-CS/GC | $-240^b$ | 0.69 | $1.74 \times 10^{-10}$ | 16 μM | 2–1100 μM | 660 nM | [5] |
| Hb/NiO/GC | $-70^a$ | 5.2 ± 0.5 | $1.73 \times 10^{-11}$ | 1370 μM | 1–2000 μM | 630 nM | [6] |
| NF/Hb/PAM-P123/GC | $-317^b$ | - | $7.64 \times 10^{-11}$ | 36 μM | 1–30 μM | 400 nM | [7] |
| Hb/Gel/GC | $-380^b$ | - | - | - | 50–1200 μM | 3400 μM | [8] |
| Hb/NGC-SF/GC | $-380^b$ | 1.98 | - | - | 0.6–1.7 and | - | [9] |
| Polymer–Hb–CNTs/GC | $-273^b$ | 0.90 | $1.1 \times 10^{-10}$ | 140 μM | 8–240 μM | 4 μM | [10] |
| Hb/CS–[bmim]PF$_6$-TiO$_2$-GR/GC | $-206^b$ | 0.73–3.96 | $3.21 \times 10^{-10}$ | 1245 μM | 1–1170 μM | 0.3 μM | [17] |
| Hb/PLGA/ILs/GC | $-318^b$ | 5.02 ± 0.16 | $4.74 \times 10^{-10}$ | 69 μM | 5–8050 μM | 0.237 μM | [21] |
| Hb/ATP/GC | $-362^b$ | 4.6 ± 0.65 | $6.7 \times 10^{-11}$ | 490 μM | 5.4–400 μM | 2.4 μM | [22] |
| EDC-Hb/CNTs/GC | $-268^b$ | 1.02 ± 0.05 | $4.7 \times 10^{-9}$ | - | 0.25–140 μM | 0.18 μM | [23] |
| Hb/BMS/Cs/GC | $-320^b$ | - | $9.34 \times 10^{-11}$ | - | 2.5–245 μM | 0.83 μM | [55] |
| Hb/GNPs/Hb/MWNT/GC | $-355^b$ | - | - | 260 μM | 0.21–3000 μM | 80 nM | [67] |

NF: Nafion; GR: graphene; PdNPs: palladium nanoparticles; CS: chitosan; NiO: nickel oxide nanoparticles; PLGA: poly lactic-co-glycolic acid; ILs: ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM]BF$_4$; BMS: bimodal mesoporous silica; PAM-P123: polyacrylamide-P123; Gel: gelatine; NGC: nanostructured gold colloid; SF: silk fibroin; EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; ATP: attapulgite; GNPs: gold colloidal nanoparticles; $^a$ vs. Ag/AgCl; $^b$ vs. SCE.
3.7. Stability

Long-term stability is an important parameter for biosensors. The operational stability of the modified electrode was determined by the CV method. The cathodic peak current was reduced by less than 5% after 50 cycles at the scan rate of 0.05 V/s, while the peak potential remained unchanged. As for the storage stability, the CVs showed minimal change after two weeks of storage in a bottle over the PBS solution at 4 °C. NF may offer a biocompatible micro-environment to confine bio-macromolecules at their ionic cluster region (30–50 nm), and this view was also consistent with our previous study [45–48]. Moreover, Naftion may be helpful to restrict, confirm and protect Hb/FMWCNTs/Cys/AuNPs system on GC electrodes.

3.8. Interference Determination

The degree of interference from interfering substances can be evaluated by the value of the cathodic current ratio which were calculated by reading the cathodic current ($I_{pc1}$) of the proposed biosensor in 50 mM PBS (pH 7.0) containing 0.10 mM H$_2$O$_2$ and a 0.20 mM hampering substance, and then, comparing it with the cathodic current ($I_{pc0}$) from the proposed biosensor in the same solution containing only 0.10 mM H$_2$O$_2$. Five interfering substances were tested here and the results are listed in Table 2. It can be observed that none of the tested interferents could cause interference to the determination of H$_2$O$_2$, which is largely attributed to the low working potential of $-350$ mV used in the determination of H$_2$O$_2$ and the negatively charged NF protection membrane.

### Table 2. Effects of possible interferences on the hydrogen peroxide biosensor.

| Possible Interferences | $I_{pc1}/I_{pc0}$ | R.S.D (%) |
|------------------------|-------------------|-----------|
| Glucose                | $1.04 \pm 0.08$   | $3.2$     |
| Adenosine Triphosphate | $1.03 \pm 0.06$   | $3.4$     |
| L-Histidine            | $1.01 \pm 0.05$   | $2.9$     |
| Ascorbic Acid          | $0.98 \pm 0.06$   | $3.1$     |
| Thiol                  | $1.00 \pm 0.04$   | $2.5$     |

$I_{pc1}$: The cathodic current for a mixture of a 0.20 mM interfering substance and 0.10 mM H$_2$O$_2$; $I_{pc0}$: The cathodic current for 0.10 mM H$_2$O$_2$ alone in a 50 mM PBS (pH 7.0), at $-350$ mV vs. Ag/AgCl. R.S.D: Relative standard deviation, obtained for nine measurements.

4. Conclusions

The direct electrochemical properties of immobilized Hb on a FMWCNTs/Cys/AuNPs-modified glassy carbon electrode were found to be due to the excellent microenvironment provided by NF, AuNPs and FMWCNTs for Hb. The small value of $K_{m}^{app}$, high sensitivity, long-term stability and low detection limit were other characteristics of the biosensor. The modified electrode showed the ability to be used as a third generation biosensor for determination of H$_2$O$_2$ at ultra-trace levels. Moreover, a redox protein on the functional nano complex modified electrode may be a new useful electrochemical tool for the analysis of relationship between the structure and function of redox proteins, especially for a heme-containing protein.
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Conflict of Interest

The authors declare no conflict of interest.

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