

# A novel biosensor based on a gold nanoflowers/hemoglobin/carbon nanotubes modified electrode

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Well-oriented 3D gold flower-like nanoparticles were successfully synthesized by a facile one-pot method, and the gold nanoflowers (AuNFs) were mixed with hemoglobin (Hb) to form a gold nanoflowers/hemoglobin composite. The composite was further combined with multiwalled carbon nanotubes on a glassy carbon electrode (GCE) to fabricate a novel biosensor. The sensor has high stability and bioactivity, and was studied by scanning electron microscopy (SEM) and cyclic voltammetry (CV). The hemoglobin/gold nanoflowers/multiwalled carbon nanotubes glassy carbon electrode (Hb/AuNFs/CNTs/GCE) either retained the Hb in similar native conformations or promoted direct electron transfer. Moreover, the sensor exhibited remarkable catalytic activity toward  $\text{H}_2\text{O}_2$  and trichloroacetic acid (TCA). The linear relationship for the determination is in the range of 1.0–60  $\mu\text{M}$  for  $\text{H}_2\text{O}_2$  and 0.06–28 mM for TCA. The detection limits were 0.08  $\mu\text{M}$  and 7.3  $\mu\text{M}$  ( $S/N = 3$ ), respectively.

## 1. Introduction

Direct electrochemistry of proteins has attracted considerable attention, because it provides fundamental knowledge of the redox behavior of proteins and is more effective to fabricate biosensors, bioreactors, and biomedical devices.<sup>1,2</sup> However, direct electron transfer of protein is generally difficult due to the embedment of the electroactive center or the denaturation of the protein adsorbed on the electrode surface. Hence various modified electrodes were developed, and the immobilization of protein has been widely studied.<sup>3–9</sup> Especially, when the nanoparticles are employed in the fabrication of biosensing interfaces, biosensors based on nanohybrids can become a promising platform for direct electron transfer. Due to the unique properties of nanoparticles, the nanostructures can offer the electron transfer interface to load biomolecules and exhibit the properties of inherent catalysis, penetrability and resistance to biodegradation.<sup>10–13</sup>

Gold nanoparticles have been widely used in protein immobilization, which act as a good conductive mediator to facilitate the electron transfer rate.<sup>14</sup> Yang *et al.* investigated the direct electrochemistry of Hb on a gold nanowire array.<sup>15</sup> Zen *et al.* combined gold nanoparticles with polyvinyl alcohol and 1-octyl-3-methylimidazolium hexafluorophosphate (OMIMPF<sub>6</sub>) for the investigation of the electrochemical behaviors of Hb.<sup>16</sup> Hong and Dai constructed an amperometric biosensor for hydrogen

peroxide and nitrite by immobilizing Hb on a one-dimensional gold nanoparticle.<sup>17</sup> Sun fabricated a modified electrode using a gold nanoparticle decorated carbon ionic liquid for direct electrochemistry and electrocatalysis of Hb.<sup>18</sup> According to our previous work,<sup>19</sup> the shaggy flower-like gold nanoparticles could provide larger surface area while retaining their native good biocompatibility.

Another long-standing goal in electrochemical biosensors is the stability and activity of the immobilized biocomponents on a solid support. Taking account of the advantages of gold nanoflowers (AuNFs) and carbon nanotubes (CNTs), the composite has more potential applications for the generation of electrochemical sensors. Thus, the importance of the interactions between CNTs and metal nanoparticles (NPs) has also been increasingly recognized due to their remarkable properties. Some groups<sup>20,21</sup> reported that the CNTs could be functionalized to attach metal NPs uniformly by virtue of the electrostatic interaction and the hybrids could be used to fabricate GNPs/CNTs/GOx multilayer composites for glucose biosensing.

Herein, a facile one-pot method, based on crystal growth in the ligand protection (LLP),<sup>22</sup> was used to fabricate Au nanoflowers (AuNFs) by using  $\text{HAuCl}_4$  and pyrrole (Py). The as-synthesized AuNFs were well-dispersed as individual entities and appeared to be strikingly uniform in morphology with the size in the range of 40–100 nm. The AuNFs displayed well-oriented 3D flower-like NPs consisting of a solid core with short, irregular, and obtuse Au nanocrystals. The roughness surface of AuNFs can provide a larger surface area and good biocompatibility. The composite can be combined with a multiwalled carbon nanotubes modified glassy carbon

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electrode (GCE) to form the Hb/AuNFs/CNTs/GC modified electrode. The electrode was characterized by scanning electron microscopy (SEM). Electrochemical impedance spectroscopy and cyclic voltammetry (CV) were employed. The direct electron transfer was realized at the modified electrode. The electrocatalytic ability of Hb in the reduction of  $\text{H}_2\text{O}_2$  and trichloroacetic acid (TCA) was further investigated.

## 2. Experimental

### 2.1. Materials and reagents

Hemoglobin (Hb, bovine blood) was obtained from Shanghai Sinopharm Chemical Reagent Co., Ltd. and used as received without further purification. Multiwalled carbon nanotubes were provided by Shenzhen Nanotech Port Co. Ltd (Shenzhen, China). All other chemicals were of reagent grade. Hydrogen peroxide was freshly prepared prior to each measurement. 0.1 M phosphate buffers with various pH were prepared by mixing the stock solutions of  $\text{K}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ . The pH values were adjusted with HCl or NaOH solution. All the aqueous solutions were prepared with Millipore water having a resistivity of 18.2 M ( $\Omega\text{-cm}$ ) (Purelab Classic Corp., USA).

### 2.2. Apparatus

SEM measurements were performed by using a Hitachi S-4800 instrument. CV and amperometric measurements were performed on a CHI 670a Electrochemical Workstation (Shanghai Chenhua Instruments Co., Ltd., China). All the electrochemical experiments were carried out in a three-electrode system, in which the modified glassy carbon electrode (GCE, 3 mm diameter) was the working electrode, a platinum wire was the auxiliary electrode and a saturated calomel electrode (SCE) was the reference electrode. Prior to the electrochemical measurements, buffer solutions were purged with high purity nitrogen for at least 20 min, and then a nitrogen environment was kept over the solution. All experiments were performed at room temperature.

### 2.3. Preparation of gold nanoflowers

In a typical synthesis, 10.0  $\mu\text{L}$  of distilled pyrrole monomer was mixed with 5 mL of SDS aqueous solution (8.27 mM, 1.0 CMC) and stirred for 2 h to obtain a uniform emulsion. 2 mL of  $\text{HAuCl}_4$  aqueous solution (14.8 mM) was added to the emulsion. Then, the mixture was allowed to react at 0–3  $^\circ\text{C}$  (in an ice bath) for 30 min under stirring. The product was washed with deionized water and ethanol several times to remove residual Py, and dried under a vacuum for 24 h. Then, the powder of AuNFs was dispersed into water before use.

### 2.4. Preparation of soluble PDDA-CNTs (PDCNTs)

CNTs were chemically shortened by ultrasonic agitation in a mixture of sulfuric acid and nitric acid (3 : 1) for 3 h. The resulting CNTs were separated and washed repeatedly with distilled water by centrifugation until pH equalled 7. The purified CNTs were functionalized with PDDA according to the following procedures: 0.5  $\text{mg mL}^{-1}$  CNTs were dispersed

into a 0.20% PDDA aqueous solution containing 0.5 M NaCl and the resulting dispersion was sonicated for 30 min to give a homogeneous black suspension. Residual PDDA polymer was removed by high-speed centrifugation and the complex was rinsed with water at least three times. The collected complex was redispersed in water with mild sonicating to produce a stable solution of the complex, which was sonicated for 5 min immediately before use.

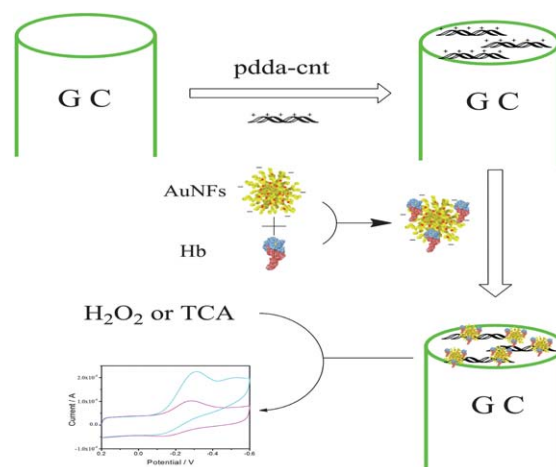
### 2.5. Electrode modification

A glassy carbon electrode (3 mm diameter) was polished successively with 1.0, 0.3, and 0.05  $\mu\text{m}$  alumina powders on silk, and rinsed thoroughly with distilled water between each polishing step. After successive sonication in 1 : 1 nitric acid, acetone, and doubly distilled water, the electrode was rinsed with doubly distilled water and allowed to dry in blowing  $\text{N}_2$ . The modification procedure was illustrated in Scheme 1: 5 ml in 5  $\text{mg mL}^{-1}$  PDCNTs solution was dropped on the pretreated GCE and dried in a silica gel desiccator, then immersed in the Hb/AuNFs solution for 60 min. After the Hb/AuNFs/CNTs/GCE was thoroughly rinsed with water, it was stored at 4  $^\circ\text{C}$  when not in use.

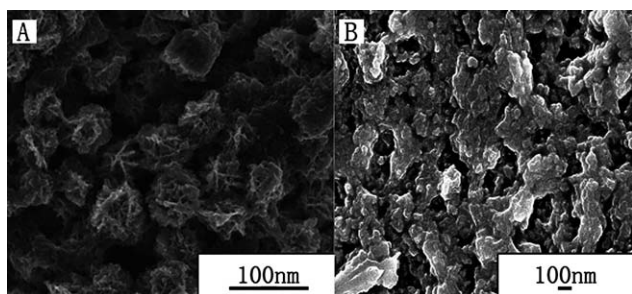
## 3. Results and discussion

### 3.1. SEM of the gold nanoflowers and Hb-gold nanoflowers composite

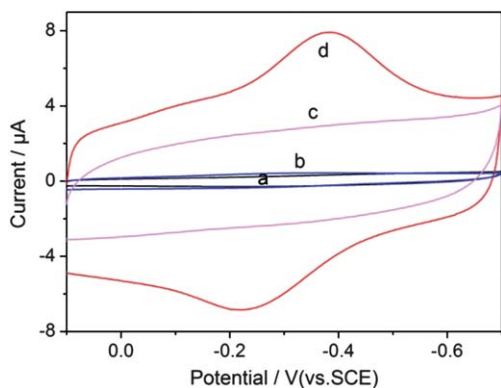
The SEM images in Fig. 1A obviously show that the synthesized nanoparticles were well-dispersed and appeared to be flower-like in morphology with the size in ranging from 40 to 100 nm. Every gold nanoflower has numerous porous structures capable of holding enzymes or substrates conjugated to enzymes intact, thereby preventing them from denaturing. In comparison, it was evident that when the gold nanoflowers were mixed with hemoglobin, the surface became smooth as shown in Fig. 1B. It could be attributed to the gold nanoflowers being combined with hemoglobin.



**Scheme 1** The modification procedure of the Hb/AuNFs/CNTs electrode.



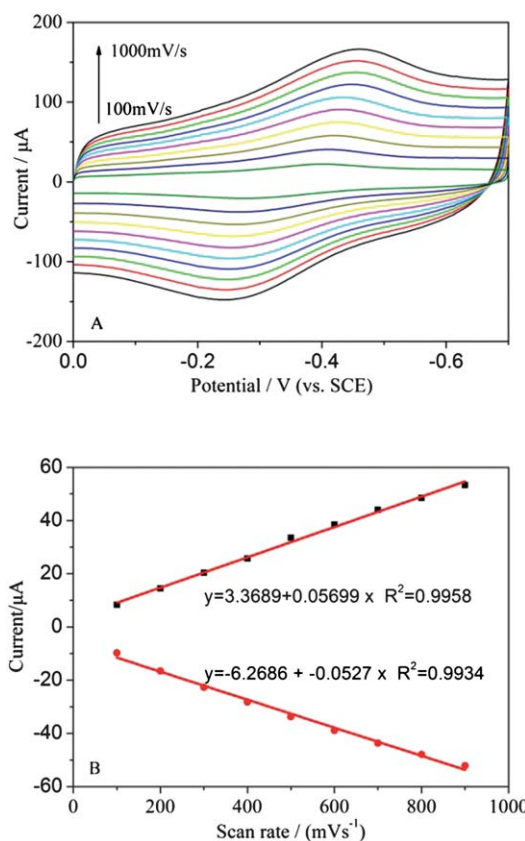
**Fig. 1** Scanning electron micrographs of gold nanoflowers (A) and Hb-gold nanoflowers composite (B).



**Fig. 2** Cyclic voltammograms of a bare GCE (a), AuNFs/GCE (b), CNTs/GCE (c) and Hb/AuNFs/CNTs/GCE (d) in 0.1 M pH 7.0 PBS at a scan rate of 100 mV s<sup>-1</sup>.

### 3.2. Cyclic voltammetric behavior of Hb/AuNFs/CNT modified glass carbon electrode

Fig. 2 shows the CVs of the modified electrodes in pH 7.0 B-R buffer solution (0.1 M). A pair of well-defined, quasi-reversible CV peaks (curve a) were observed for the Hb/AuNFs/CNT modified glassy carbon electrode with anodic peak potential ( $E_{pa}$ ) of  $-271$  mV and cathodic peak potential ( $E_{pc}$ ) of  $-410$  mV at a scan rate of 100 mV s<sup>-1</sup>. The formal potential ( $E^{\circ}$ ), which was calculated from the midpoint of the reduction and oxidation peak potentials, was  $-340$  mV (vs. SCE). The peak-to-peak potential difference ( $\Delta E_p$ ) was 140 mV, indicating that Hb immobilized on the Hb/AuNFs/MCNT/GC electrode surface, this is a one-electron quasi-reversible electrochemical reaction. The peaks came from heme Fe(III)/Fe(II) redox couples of proteins. While, no voltammetric peaks were observed at the AuNFs/GC (curve b) and MCNT/GC (curve c) electrodes in the same potential range. As we know, the Hb could also have direct electron transfer on the GCE, but only small redox peaks can be observed, so the presence of gold nanoflowers could greatly promote the electron-transfer of Hb. For the Hb/AuNFs/CNTs/GC electrode, the peak current reached steady state after scanning several cycles. Thus, all of the CV experiments on the Hb/AuNFs/CNTs/GCE electrode were conducted at their steady states. Fig. 3A shows the CVs of the Hb/AuNFs/CNTs/GC electrode in pH 7.0 PBS buffer solution with different scan rates. The results indicated that the electroactive Hb Fe(III) in the film was reduced to Hb Fe(II) on the forward scan, and then



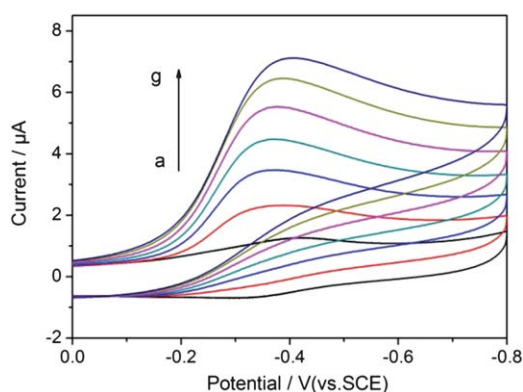
**Fig. 3** (A) Cyclic voltammograms of the Hb/AuNFs/CNTs/GC electrode in pH 7.0 PBS at 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mV s<sup>-1</sup> (from inner to outer). (B) shows the dependence of the anodic and cathodic peak currents on the scan rate.

reoxidized to Hb Fe(III) on the reverse scan. The reduction ( $I_{pc}$ ) and oxidation ( $I_{pa}$ ) peak currents exhibited a linear relationship with the scan rate ( $\nu$ ) in the range from 100 to 1000 mV s<sup>-1</sup>. Furthermore,  $I_{pc}-\nu$  and  $I_{pa}-\nu$  plots were linear (Fig. 3B). These results indicated the characteristics of quasi-reversible surface controlled thin-layer electrochemical behavior.

### 3.3. Electrocatalytic reduction of hydrogen peroxide

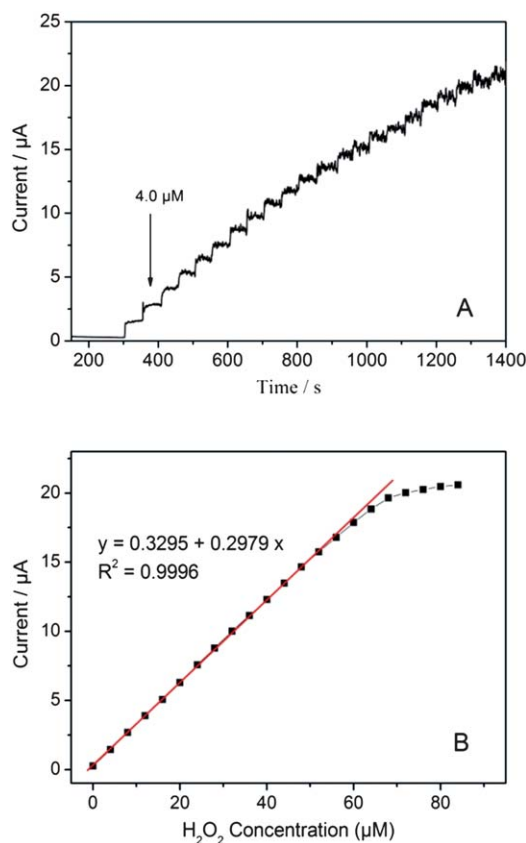
Electrochemical catalytic reduction of H<sub>2</sub>O<sub>2</sub> by the Hb/AuNFs/MCNTs/GC electrode was investigated by CV. After H<sub>2</sub>O<sub>2</sub> was added to the pH 7.0 B-R buffer solution, a new reduction peak appeared at about  $-380$  mV in contrast to that reported previously as shown in Fig. 4. The new peak is original from the reduction of H<sub>2</sub>O<sub>2</sub>.

The Hb/AuNFs/CNTs/GC modified electrode showed a more sensitive response to H<sub>2</sub>O<sub>2</sub> than only MCNT or Au nanoflower modified electrodes. The Hb/Au modified electrode showed a good catalytic response to the reduction of H<sub>2</sub>O<sub>2</sub>. Upon addition of an aliquot of H<sub>2</sub>O<sub>2</sub> to the buffer solution, the reduction current increased greatly, and then reached a stable value. The anodic peak current decreased and disappeared after the addition of a limiting concentration of H<sub>2</sub>O<sub>2</sub> into the buffer solution. This is the characteristic of an enzymatic catalysis. The amperometric response of the Hb/Au modified electrode for successive addition of H<sub>2</sub>O<sub>2</sub> to 0.1 M pH 7.0 PBS at an applied



**Fig. 4** Cyclic voltammograms of Hb/AuNFS/CNTs/GC in pH 7.0 PBS after the addition of 0.0,  $1.0 \times 10^{-5}$ ,  $2.0 \times 10^{-5}$ ,  $3.0 \times 10^{-5}$ ,  $4.0 \times 10^{-5}$ ,  $5.0 \times 10^{-5}$ ,  $6.0 \times 10^{-5}$ , mol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub> (a–g), respectively, at the scan rate of 100 mV s<sup>-1</sup>.

potential of  $-400$  mV is shown in Fig. 5. The peak current was linearly proportional to the concentration of H<sub>2</sub>O<sub>2</sub>. The linear regression equation was  $I_p$  (µA) = 0.2979 C<sub>H<sub>2</sub>O<sub>2</sub></sub> (µM) + 0.3295 (µA) in the range from 1.0 to 60 µM with a correlation coefficient of 0.9996 ( $n = 5$ ) and a detection limit of 7.3 µM when the signal-to-noise ratio was 3. When the concentration of H<sub>2</sub>O<sub>2</sub> was higher than 80 µM, a platform was observed, indicating a saturation of the enzyme substrate reaction. The apparent Michaelis–Menten

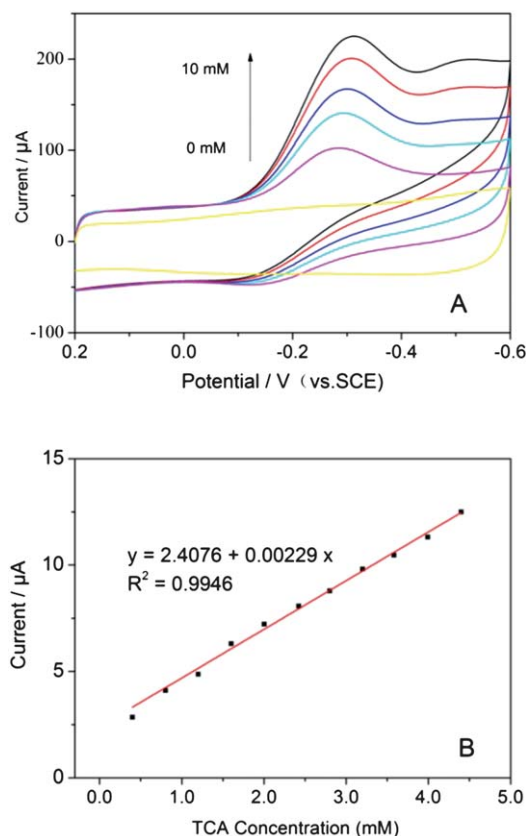


**Fig. 5** (A) The amperometric response of the sensor with successive additions of  $4 \times 10^{-6}$  M H<sub>2</sub>O<sub>2</sub> in 0.1 M pH 7.0 PBS at an applied potential of  $-0.40$  V. (B) shows the linear dependence of the reduction peak current on the H<sub>2</sub>O<sub>2</sub> concentration.

constant ( $K_m^{app}$ ), can be calculated using to the Lineweaver–Burk equation.<sup>23</sup> The  $K_m^{app}$  value for the Hb/AuNFS/CNTs/GCE electrode-based H<sub>2</sub>O<sub>2</sub> sensor was calculated as 10.35 µM, smaller than that in mesoporous molecular sieves.<sup>24</sup> The smaller  $K_m^{app}$  meant that the present Hb exhibited higher affinity to H<sub>2</sub>O<sub>2</sub>.

### 3.4. Electrocatalytic reduction of trichloroacetic acid (TCA)

TCA is an analogue of acetic acid in which the three hydrogen atoms of the methyl group have all been replaced by chlorine atoms. It is widely used in the precipitation of macromolecules, such as proteins, DNA and RNA, and in cosmetic treatments, such as chemical peels and tattoo removal. It is also an environmental pollutant which can kill normal cells. For these reasons, the determination of TCA has received great attention in recent years. The electrochemical catalytic reduction of TCA was investigated by CV. When TCA was added to a pH 7.0 buffer, a new reduction peak appeared at about  $-290$  mV as shown in Fig. 6. Similar to the behavior of the Hb/AuNFS/CNTs/GCE electrode to H<sub>2</sub>O<sub>2</sub>, the oxidation peak current increased with the addition of TCA. The results indicated that the Hb Fe(II) was chemically oxidized by TCA and the produced Hb Fe(III) was reduced again at the electrode in a catalytic cycle. Catalytic reduction of TCA by Hb can be expressed by the following equations:<sup>25,26</sup>



**Fig. 6** (A) Cyclic voltammograms obtained at Hb/AuNFS/CNTs/GC in pH 7.0 PBS after the addition of 0,  $2.0 \times 10^{-3}$ ,  $4.0 \times 10^{-3}$ ,  $6.0 \times 10^{-3}$ ,  $8.0 \times 10^{-3}$ , and  $1.0 \times 10^{-2}$  mol L<sup>-1</sup> TCA, respectively, at the scan rate of 100 mV s<sup>-1</sup>. (B) shows the linear dependence of the reduction peak current on the TCA concentration.



The Hb Fe(III) reduction peak current was linearly proportional to the concentration of TCA. Fig. 6B shows the linear dependence of the reduction peak current on the TCA concentration. The linear regression equation was  $I_p$  ( $\mu\text{A}$ ) =  $0.00229C_{\text{TCA}}$  (mM) + 2.4076 ( $\mu\text{A}$ ) in the range from 0.06 to 28 mM with a correlation coefficient of 0.9946 ( $n = 5$ ). The detection limit is 7.3  $\mu\text{M}$  when the signal-to-noise ratio was 3. However, no corresponding electrochemical signal could be observed in the same potential window when a bare CNTs/GCE or AuNFs/GCE electrode were employed in the same TCA solution. Therefore, the catalytic process was a result of the specific enzymatic catalytic reaction between Hb and TCA. When the concentration of TCA was higher than 40 mM, the response showed a levelling-off tendency, indicating a saturation of the enzyme substrate reaction.

#### 4. Conclusions

In this paper, a Hb/AuNFs/CNTs/GC electrode was constructed using the film deposition method. The properties of Hb in the Hb/AuNFs/CNTs/GCE electrode were characterized with scanning electron microscopy and an electrochemical method, and it was found that the Hb retained a nearly native secondary structure in the support medium. The Hb immobilized on the Hb/AuNFs/CNTs/GC electrode displayed excellent electrocatalytic activity in the reduction of  $\text{H}_2\text{O}_2$  and TCA.  $\text{H}_2\text{O}_2$  had a linear current response from 1.0 to 60  $\mu\text{M}$  ( $R^2 = 0.9996$ ;  $n = 5$ ) with a detection limit of 0.08  $\mu\text{M}$  when the signal-to-noise ratio was 3. Similarly, TCA had a linear current response from 0.06 to 28 mM. The detection limits were 7.3  $\mu\text{M}$  when the signal-to-noise ratio was 3. The results indicated that the peak potentials and currents of the Hb/AuNFs/CNTs/GCE were stable for 4 weeks and then decreased gradually. The direct immobilization of proteins onto the surface of AuNFs/CNTs/GCE is shown to be a highly efficient method for the development of a new class of very sensitive, stable, and reproducible electrochemical biosensors.

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