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# Hydrogen Peroxide Biosensor Based on Enzymatic Modification of Electrode Using Deposited Silver Nano Layer

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In this work, a new biosensor based on horseradish peroxidase (HRP) immobilized on a silver nano-layer modified glassy electrode denoted as HRP/AgNL/GCE, was studied. The resulted biosensor exhibits enhanced electrocatalytic activity toward reduction of $H_2O_2$. Ag nano-layer was shown to enhance the electron transfer as a mediator and capable to carry higher bioactivity owing to its intensified surface area. Obtained electrode biosensor exhibited a detection limit of 0.13μM, and a linear range of $3.85\times10^{-5}$ to $5.2\times10^{-4}$ M at a signal-to-noise ratio of 3 .

**Keywords:** HRP, Hydrogen peroxide, Ag nano-layer, cyclic voltammetry

## 1. INTRODUCTION

Nano-based materials, e.g. nanoparticles, nanotubes and nanocomposites, have been used for the manufacture of electrochemical sensing and biosensing devices with excellent analytical performances, thanks to their small sizes, large surface-to-volume ratios and increased surface activities [1].

Also it is well-known that, mediators are widely used to act as the charge carriers and can decrease the effects of interferences, lower the operating potential of the electrodes, and improve the linear response range and sensitivity of the sensor [2].

There are various method for enzyme immobilization on surface of electrodes including covalent bonded, physical adsorption, cross-linked, embed (enzyme into gels, cross-linked polymers), electrochemical deposition, electrostatic adsorption, etc [3].

Hydrogen peroxide is one of the significant intermediate in bio systems and its determination is important in various aspects.

One of the most important of use of $H_2O_2$ analysis is Glucose analysis which generally performed by quantity of the current associated with the oxidation hydrogen peroxide, a side product in the course of the enzymatic reaction. However, a great problem for this purpose is that high over voltage needed for hydrogen peroxide oxidation which in this large over-potential there are some electroactive substances, such as ascorbate and urate present in real samples could be electrochemically oxidized to give interfering signals.

Several techniques have been proposed to suppress the anodic discharge of interferences for example using nafion as negatively charged membranes on the electrode tip to force out negatively charged interference [4] or selective catalysts to lower or diminish the overpotential for the anodic oxidation of hydrogen peroxide, such as metallic particles [5].

Also there is another way to achieve this purpose by cathodic reduction of hydrogen peroxide promoted by some electron mediators, such peroxidase at lower overpotential where the reduction of dioxygen can be circumvent at the same time [6].

For cathodic reduction of $H_2O_2$ horseradish peroxidase (HRP) is an important heme containing enzyme that has been studied for more than one century [7]. Usually, leak of small HRP into bulk solution is possible. Also denaturation and loss of bioactivity of the enzyme will occurred in the case of direct adsorption of HRP onto bare electrode. Therefore, development of a simple and stable immobilization method is of great importance.

As we know it is possible to deposit Ag nanoparticle to form Ag nano layer denoted as AgNL on bare electrode by applying certain potential to electrode in $AgNO_3$ solution. Welch et al. [8] have explored the fabrication of silver nanoparticles deposited on to a glassy carbon surface for the electro-analytical detection of hydrogen peroxide.

They founded that the magnitude of the current is increased with addition of hydrogen peroxide. They resulted that this is due to the electrochemical reduction of hydrogen peroxide at the silver nanoparticle sites.

To the best of our knowledge, nafion/HRP/AgNL/GCE has not been utilized so far based of electrochemical deposition of silver for the fabrication of a $H_2O_2$ biosensor. Silver nanoparticles as nano layer became our research target due to their excellent conductivity, and the synthesis of silver nanoparticles to form nano-layer has been well-demonstrated. HRP was selected as a model because it is well-studied and it is commercially available in a highly purified form [9].

In this paper, firstly we applied a constant potential to bar GCE for participate of Ag nanoparticle and formation of a nano layer (AgNL), then we immobilized HRP to form HRP/AgNL/GCE. Finally diluted nafion used to cover biosensor and prevent the enzyme leakage. The electrocatalytic response of resulted biosensor toward reduction of $H_2O_2$ studied via cyclic voltammetry.

## 2. EXPERIMENTAL

### *2.1. Materials*

Horseradish peroxidase (EC 1.11.1.7), Nafion (5% ethanol solution) and finer alumina powder were purchased from Sigma, St. Louis, USA. Aqueous solution of hydrogen peroxide (30%),di-potassium hydrogen phosphate and potassium di-hydrogen phosphate were purchased from Merck( Germany). All of other reagent such as silver nitrate and potassium nitrate purchased from Fluka (Switzerland) Additions of hydrogen peroxide were made from a 10 mmol $L^{-1}$ stock solution, prepared by dilution from a solution obtained from Aldrich (30% w/w in water, 99% purity). The stock solution was prepared daily to avoid excessive decomposition of the hydrogen peroxide to water and oxygen, which is accelerated when the solution is diluted. After each addition the cell solution was stirred using a magnetic stirrer.

All experiments were carried out in a cell of volume 10 ml under $N_2$ saturated condition and at the room temperature (25℃).

### *2.2. Apparatus*

Electrochemical experiments were performed by using a Ivium state (A08085) electrochemical analyzer (the Netherlands). A GC working electrode (2mm diameter), a Platinum wire counter electrode, saturated calomel as reference electrode and a conventional three-electrode electrochemical cell were obtained from the company of Azar electrode (Iran).

The phosphate buffer was adjusted to pH 7. Before each electrochemical measurement, the experimental solution in the cell was purged with highly purified nitrogen gas for at least 5 min to remove dissolved oxygen.

The atomic force microscopy (AFM) image was obtained using Nanoscope IIIa (Digitao Instruments, Inc., Santa Barbara. CA).

### *2.3. Preparation biosensor*

Before the surface modification, the 2-mm bare glassy carbon electrode was polished with slurries of fine alumina powders 1, 0.3 and 0.05 μm respectively on a polishing microcloth pad and washed with deionized water several times after sonication for 10 minutes.

A solution consisting of 1 mmol $L^{-1}$ silver nitrate and 0.1 mol $L^{-1}$ potassium nitrate was prepared in deionized water. After degassing with nitrogen for 10 min the silver was deposited for 1 min at -0.5 V vs. calomel, using a silver wire as the counter electrode. The HRP/AgNL/GCE was prepared by casting a 4μL of HRP ($5 mg mL^{-1}$) onto the surface of glassy carbon electrode and drying under room temperature in the air. Next, a 4μL of 0.5 wt% Nafion was coated onto the surface of HRP/AgNL/GCE.

The prepared Nafion/HRP/AgNL/GC modified electrode was dried at room temperature and used for electrochemical investigations or stored at 4 ◦C when not in use.

## 3. RESULT AND DISCUSSION

### *3.1. Characterization of biosensor*

EIS measurements give information on the impedance changes of the electrode surface. The high-frequency region contains information of kinetics of the faradaic process, while the low-frequency region gives information concerning the diffusion of species to electrode surface. The semicircle diameter of well conducting substrates equals the electron transfer resistance, Rct. If the substrate is covered by a film with some ohmic resistance, $R_f$, the diameter of the semicircle will also be dependent on that resistance [10].

Changes of the impedance spectra were observed in the course of stepwise modification of the electrode, as shown in Fig. 1, and the Ret values of the bare GCE (curve a) were estimated to be 3000Ω. When the AgNL (curve b) and HRP (curve c) were modified on the bare GCE, their Ret values increased to 6000 and 9400 Ω, respectively, These results show that presence of HRP obstructed the electron transfer of the electrochemical probe to the electrode substrate. Therefore, we can conclude that HRP has successfully been adsorbed on the surface AgNL/GCE.

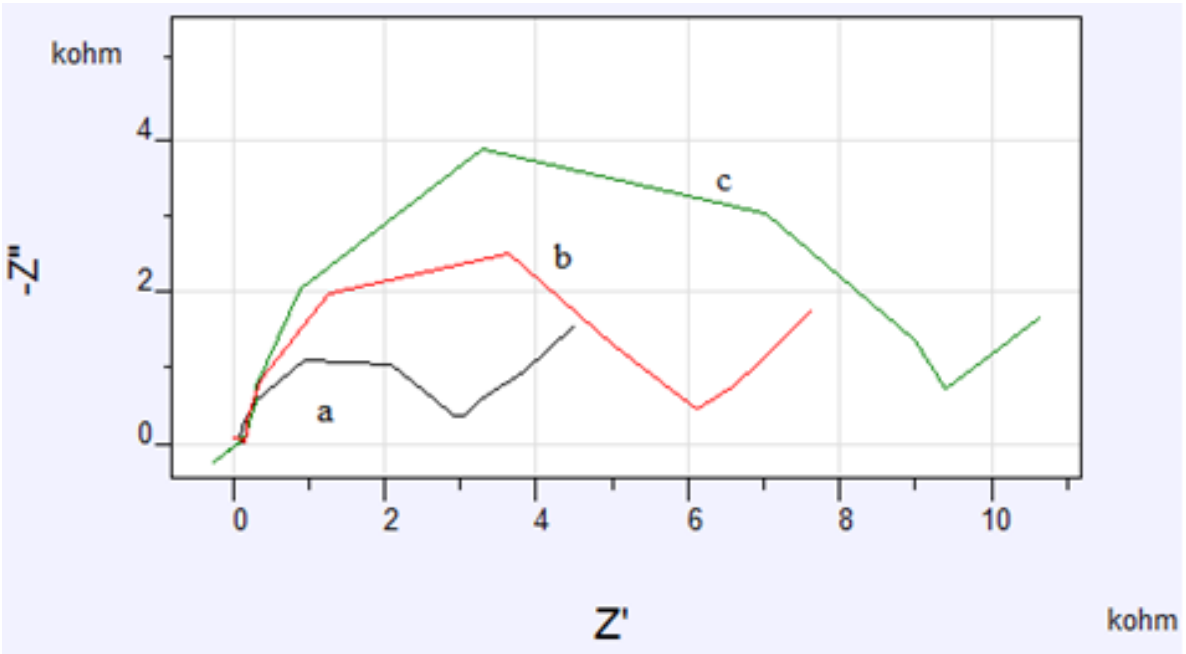


**Figure 1.** EIS of a)bare GC electrode, b) GC-Ag nano layer, c) GC-Ag nano layer-HRP

Surface morphology of AgNL/GCE (fig. 2A) and HRP/AgNL/GCE (fig. 2B) was analyzed by using atomic force microscopy (AFM). The average particle size of silver was 40 – 50 nm. Surface morphology of Ag nanoparticle is looking uniform. The thickness of film was measured by using AFM cross sectional analysis and was found 56 nm for AgNL.. As shown in fig.2, uniformly particle size disappeared and emerged long attached material which attribute to HRP enzyme.

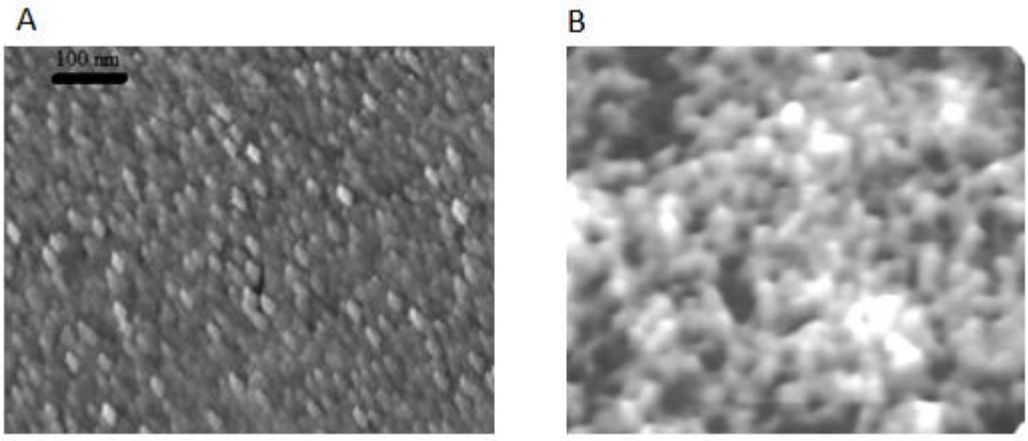


**Figure 2.** AFM images of A) AgNL/GCE , B)HRP/AgNL/GCE

*3.2. Performance of Biosensor*

To confirm whether the current was attributed to the enzymatic or nonenzymatic reduction of $H_2O_2$, A control experiment was performed using cyclic voltammetry.

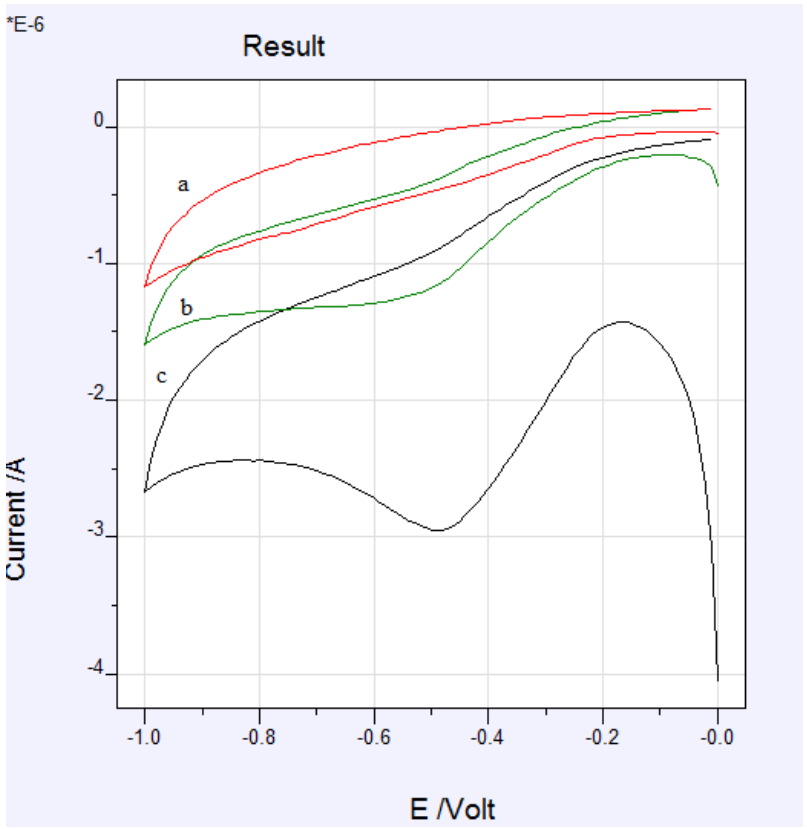


**Figure 3.** Cyclic voltammetry response of different modified electrode to $1.67 \times 10^{-4}$ $molL^{-1}$ of $H_2O_2$ in PB solution (pH =7): a) nafion/HRP/GCE, b) nafion/AgNL/GCE and c) nafion/HRP/AgNL/GCE. Scan rate: $100mVs^{-1}$

No current response appears with regarding to GC bare electrode (data not shown). As shown in Fig. 3, there were no noticeable catalytic current responses to $H_2O_2$ at the nafion/HRP/GCE (Fig. 3a) and there is slightly peak current at nafion/AgNL/GCE (Fig. 3b) which is very slight compared to that at the nafion/HRP/AgNL/GCE (Fig. 3c).

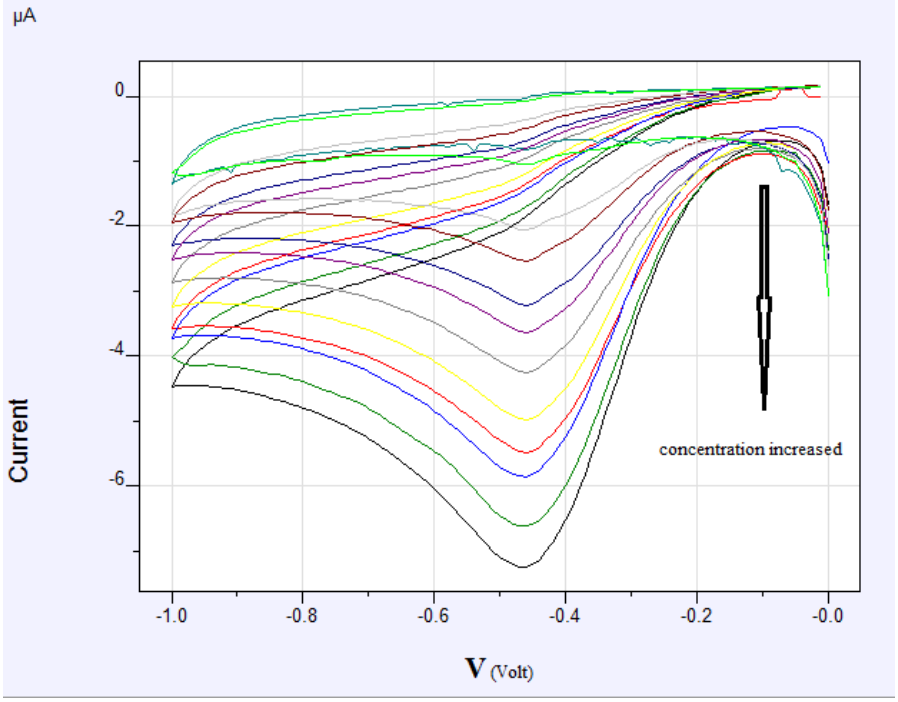


**Figure 4.** Cyclic voltammograms for the nafion/HRP/AgNL/GCE in 0.1 M PB (pH 7.0) containing from 0 to 5.23 × $10^{-4}$ $molL^{-1}$ $H_2O_2$. Scan rate: 10 mV $s^{-1}$

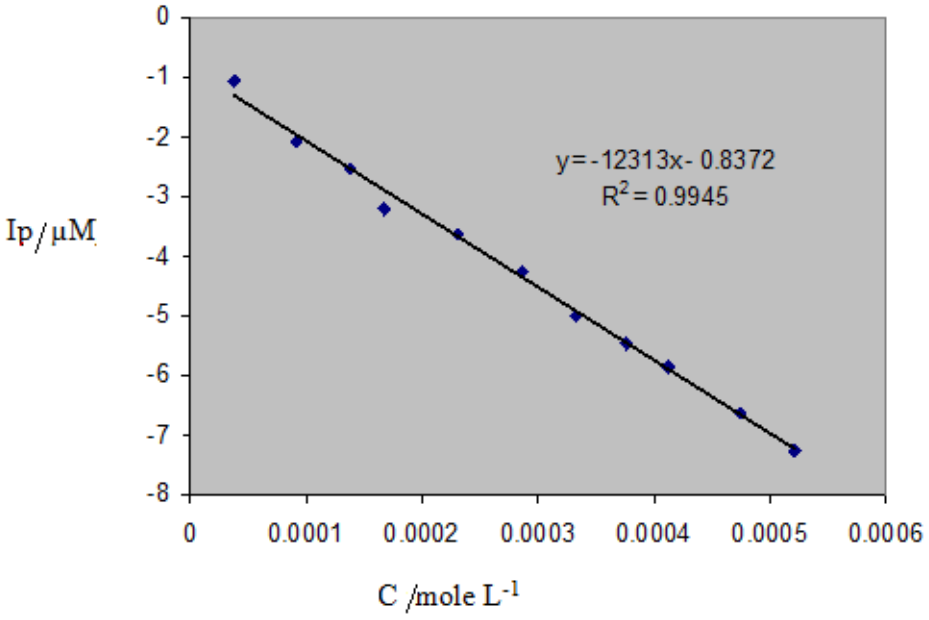


**Figure 5.** Calibration curve for obtained biosensor toward $H_2O_2$ analysis

From these results, we can result that the catalytic current was chiefly attribute to the HRP reduction of $H_2O_2$, in which the silver nanoparticles play an important role in the catalysis of this process.Cyclic voltammetry was used to study the electrocatalytic behavior of the reduction of hydrogen peroxide at the Nafion/HRP/AgNL/GC modified electrode. Figure 4 shows cyclic voltammograms for the Nafion/HRP/AgNL/GC in pH 7.0 PB containing different concentrations of $H_2O_2$. The catalytic current increased as more $H_2O_2$ was added, suggesting that the catalytic current was determined by the concentration of $H_2O_2$. The corresponding calibration curve shown in Fig. 5 pointed to that the hydrogen peroxide biosensor exhibited a detection limit of 0.13μM, and a linear range of $3.85\times10^{-5}$ to $5.2\times10^{-4}$ M. These observations indicate that the electrocatalytic reduction of hydrogen peroxide is achieved by HRP immobilized at Nafion/HRP/AgNL/GCE.

The mechanism of the electrocatalytic reduction of hydrogen peroxide at Nafion/HRP/AgNL/GC nanobiocomposite can be expressed as follows [11]:

$$\text{HRP-Fe(III)} + \text{H2O2} \rightarrow \text{Compound I} + \text{H2O} \quad (1)$$

$$\text{Compound I} + e^- \rightarrow \text{Compound II} \quad (2)$$

$$\text{Compound II} + e^- \rightarrow \text{HRP-Fe(III)} \quad (3)$$

In the presence of $H_2O_2$, the HRP–Fe(III) immobilized on the biosensor surface was oxidized by $H_2O_2$ to form an intermediate (compound I in Eq. 1), which is a two-equivalent oxidized form containing oxyferryl heme (Fe4+==O) and a porphyrin π cation radical. Compound I has catalytic activity; its porphyrin radical obtains one electron from the electrode to form a second intermediate (compound II) directly or via other media (Eq. 2). Compound II is subsequently reduced back to the native HRP by accepting one electron from the electrode (Eq. 3)

Fig. 8 represents the Lineweaver–Burk plot for nafion/HRP/AgNL/GCE in the presence of different concentrations of $H_2O_2$ in 0.1M PBS at pH 7.0. The apparent Michaelis–Menten constant ($K_m^{app}$) is a reflection of both the enzyme affinity and the ratio of microscopic kinetic constants. It can be observed from the electrochemical version of the Lineweaver–Burk equation:

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_m^{app}}{I_{max}C} \quad (4)$$

where Iss is the steady-state current after the addition of substrate, C the substrate concentration in the bulk solution and $I_{max}$ is the maximum current measured under saturated substrate conditions. A low $K_m^{app}$ value indicates a strong substrate binding. The $K_m^{app}$ value for HRP/AgNL/GC electrode was calculated to be 0.32 mM, which is lower than the reported value 5.5mM for the HPR-modified electrode [12] 0r 0.39mM for Nafion/HRP/MWCNT/ACS [13] giving an evidence for the higher sensitivity of nafion/HRP/Ag-nanolayer/GC electrode. These comparisons indicate that not only the HRP molecules are immobilized on GC electrode but also their affinities for $H_2O_2$ were increased.

Table 1 shows various literature limits of detection values. It can be seen that the limit of detection attained here is favorable compared with those of other methods.

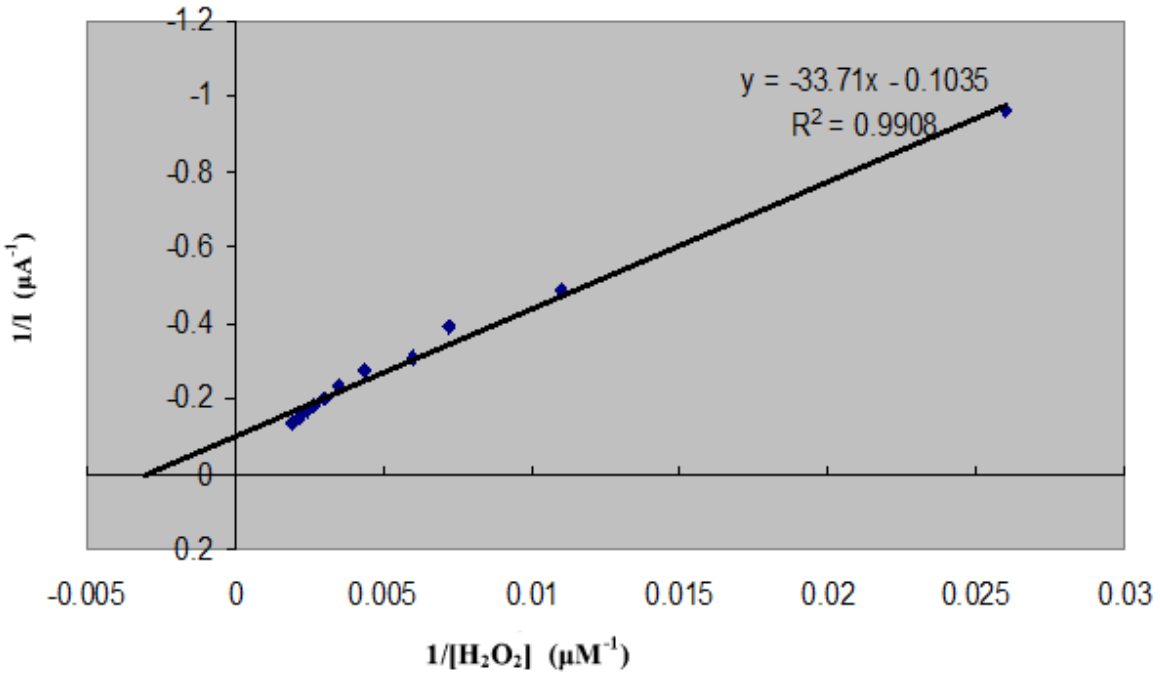


**Figure 8.** Lineweaver–Burk plot for nafion/HRP/AgNL/GCE in the presence of different concentrations of $H_2O_2$

*3.3 Stability*

**Table 1.** Comparison operation of various $H_2O_2$ sensors

| Type of sensor | LOD (μM) | Linear range (mM) | Reference |
|---|---|---|---|
| AgNPs-NFs/GCE | 62 | 0.1-70 | 14 |
| Ag/GN-R/GCE | 28 | 0.1-40 | 15 |
| Roughed Ag Electrode | 6 | 0.01-22.5 | 16 |
| AgNPS/PVA/Pt | 1.0 | 0.04-6 | 17 |
| PQ11-AgNPs/GCE | 33.7 | 0.1-180 | 18 |
| HRP/DNA–Ag/PDDA–Au/DNA–Ag/Au | 2 | 0.007- 7.8 | 19 |
| Ag microspheres/GCE | 1.2 | 0.25-2 | 20 |
| AgNPs/collagen/GCE | 0.7 | 0.005-40.6 | 21 |
| AgNPs/DNA/GCE | 1.7 | 0.004–16 | 22 |
| AgNPs/GCE | 2 | - | 23 |
| AgNPs/SBA-1S/GCE | 12 | 0.049–970 | 24 |
| AgNP–CPNBs | 0.9 | 0.1-7 | 25 |
| This work(nafion/HRP/AgNL/GCE) | 0.13 | 0.03-0.5 | - |

Further studies showed that this biosensor is relatively stable. When it was stored at 4 ºC for two weeks and used for measurements intermittently, the sensor retained more than 82.0% of its initial response to the reduction of 0.16 mM $H_2O_2$. This long-term stability can be attributed to the strong interactions between the silver nanoparticles and HRP. However, it was founded in our experimental when the concentration of $H_2O_2$ is higher than 0.5 mM, the immobilized HRP is denaturedand irreversibly transformed to its oxidized, inactive form.

*3.4 Interference study*

The selectivity of the hydrogen peroxide biosensor was investigated in the presence of some potentially coexisting interference such as uric acid (UA), ascorbic acid (AA), dopamine (DA), and glucose in biological systems. There were no obvious responses which can be seen after the additions of 0.2mM ascorbic acid, uric acid, and dopamine, respectively into 0.1mM hydrogen peroxide in 0.1M phosphate buffer solution (pH= 7). This result demonstrated that the prepared hydrogen peroxide biosensor based on Nafion/HRP/AgNL/GCE nanobiocomposite exhibited the ability to reduce the influence of possible interferences.

## 4. CONCLUSION

The Ag nanoparticles facilitate electron transfer between HRP and the bulk electrode surface and provide more binding sites for the immobilization of HRP, thus increasing the biosensor's sensitivity to the reduction of $H_2O_2$. The biosensor exhibits high stability and good sensitivity. In addition, it was easy to fabricate and was stable over prolonged use.

## References

1. L. Zhu, R. Yang, J. Zhai and Ch. Tian, *Biosens. Bioelectron*, 23 (2007) 528.
2. Zh. Wang, M. Li, P. Su , Y. Zhang, Y. Shen, D. Han, A. Ivaska and L. Niu, *Electrochem. Commun*, 10 (2008) 306.
3. A.Shokuhi Rad, A. Mirabi, E. Binaian and H. Tayebi, *Int. J. Electrochem. Sci*., 6 (2011) 3671.
4. S.H. Lim, J. Wei, J.Y. Lin, Q.T. Li, Y.J. Kua, *Biosens. Bioelectron*, 20 (2005) 2341.
5. C.X. Lei, H. Wang, G.L. Shen and R.Q. Yu, *Electroanalysis*, 16 (2004) 736.
6. J.Z. Xu, J.J. Zhu, Q. Wu, Z. Hu and H.Y. Chen, *Electroanalysis*, 15 (2003) 219.
7. N.C. Veitch, Phytochemistry, 65 (2004) 249.
8. C. M. Welch, C. E. Banks, A. O. Simm and R.G. Compton, *Anal Bioanal Chem*, 382 (2005) 12.
9. Ch. Ren, Y. Song, Zh. Li, G. Zhu, *Anal Bioanal Chem*, 381 (2005) 1179.
10. Zh. Wang, M. Li, P. Su, Y. Zhang, Y. Shen, D. Han, A. Ivaska and L. Niu, *Electrochem. Commun*, 10 (2008) 306.
11. J.L. Huang and Y.Ch. Tsai, *Sensors and Actuators* B, 140 (2009) 267.
12. T. Ferri, A. Poscia and R. Santucci, *Bioelectrochem. Bioenerg*., 45 (1998) 221.
13. J.L. Huang and Y.Ch. Tsai, *Sensors and Actuators* B, 140 (2009) 267.
14. X. Cui, G. Liu and Y. Lin, *J. Biomed. Nanotechnol*., 1 (2005) 1.

15. S. Liu, J. Tian, L. Wang, H. Li, Y. Zhang and X. Sun, *Macromolecules*, 43 (2010) 10078.
16. W. Lian, L. Wang, Y. Song, H. Yuan, S. Zhao, P. Li and L. Chen, *Electrochim. Acta*, 54 (2009) 4334.
17. M.R. Guascito, E. Filippo, C. Malitesta, D. Manno, A. Serra and A. Turco, *Biosens. Bioelectron.*, 24 (2008) 1057.
18. W. Lu, F. Liao, Y. Luo, G. Chang and X. Sun, *Electrochim. Acta*, 56 (2011) 2295.
19. L. Ma, R. Yuan, Y. Chai, Sh. Chen, *Journal of Molecular Catalysis B: Enzymatic* 56 (2009) 215.
20. B.Zhou, Z. Liu, Z. Liu, G. Liu, Z. Li, J. Wang and X. Dong, *Electrochem. Commun.*, 11 (2009) 1707.
21. Y. Song, K. Cui, L. Wang and S. Chen, *Nanotechnology*, 20 (2009) 105501.
22. K. Cui, Y. Song, Y. Yao, Z. Huang and L. Wang, *Electrochem. Commun.*, 10 (2008) 663.
23. C. Welch, C. Banks, A. Simm and R. Compton, *Anal. Bioanal. Chem.*, 382 (2005) 12.
24. D.H. Lin, Y.X. Jiang, Y. Wang, S.G. Sun, *J. Nanomater.*, 1 (2008) 473791.
25. Y. Luo, W. Lu, G. Chang, F. Liao and X. Sun, *Electrochimica Acta*, 56 (2011) 8371.